CD63 EXPRESSION ON BASOPHILS IN PATIENTS WITH ALLERGY TO BETA-LACTAM ANTIBIOTICS

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CD63 EXPRESSION ON BASOPHILS IN PATIENTS WITH ALLERGY TO BETA-LACTAM ANTIBIOTICS

ABSTRACT

Immediate-type hypersensitivity reactions to beta-lactam antibiotics are an increasing clinical issue. However, diagnosis is challenging with little information from current available *in vivo* and *in vitro* tests. The gold standard test for diagnosis of drug allergy is the risky drug provocation test performed under close clinical supervision. In the current study, we aimed to study the expression of CD63 marker on basophil cells in patients with beta-lactam allergy, thus identify if this test can be useful in preventing misdiagnosis in these patients. We recruited 25 patients with suggestive clinical characteristics of allergy to beta-lactam antibiotics and 25 healthy controls. Skin Prick Test (SPT) using a panel of beta-lactam allergens consists of Penicilloyl-polylysine-Minor Determinant Mix (PPL-MDM), Amoxicillin and Clavulanic Acid were carried out in 24 patients. CD63 expression was determined by employing Basophil Activation Test (BAT) using Penicillin G, Penicillin V, Penicilloyl-polylysine (PPL), Minor Determinant Mix (MDM), Ampicillin and Amoxicillin, along with specific IgE quantification by Fluorescence Enzyme Immunoassay (FEIA) using Penicillin G, Penicillin V, Ampicillin and Amoxicillin in all participants. Of 24 patients, one patient was SPT-positive to Amoxicillin and in-house Ampicillin preparation. Two patients were BAT-positive and four patients were FEIA-positive. One patient showed consistent result in BAT and SPT while another patient showed consistent result in BAT and FEIA. We observed fair agreement between BAT and FEIA (Cohen Kappa Index=0.25). Although the agreement between CD63 expression in BAT and FEIA is fair, their diagnostic values are complementary. Despite good specificity, both tests

demonstrated low sensitivity. BAT is particularly useful in patients with a clinical history of anaphylaxis and negative for FEIA, thus may avoid risky drug provocation test.

EKSPRESI CD63 PADA BASOFIL UNTUK ALAHAN TERHADAP ANTIBIOTIK BETA-LAKTAM

ABSTRAK

Reaksi hipersensitiviti jenis segera terhadap antibiotik beta-laktam merupakan isu klinikal yang semakin meningkat. Walau bagaimanapun, diagnosis adalah mencabar dengan informasi terhad yang diperolehi daripada ujian *in vivo* dan *in vitro* sekarang. Ujian piawai emas untuk mengenal pasti alahan dadah adalah ujian provokasi dadah berisiko tinggi yang perlu dijalankan di bawah penyeliaan klinikal yang ketat. Di dalam kajian ini, kami bertujuan untuk mengkaji ekspresi penanda CD63 pada sel basofil di kalangan pesakit yang mempunyai alahan kepada beta-laktam, dan seterusnya mengenal pasti samaada ujian ini boleh digunakan untuk mengelakkan misdiagnosis di kalangan pesakit tersebut. Kajian ini melibatkan, seramai 25 pesakit yang mempunyai karakter klinikal cenderung kepada alahan terhadap antibiotik beta-laktam dan 25 individu sihat. Ujian Cucuk Kulit (SPT) yang menggunakan panel alergen beta-laktam yang terdiri daripada Campuran Penentu Utama dan Penentu Kecil (PPL-MDM), Amoksisilin dan Asid Klavulanik telah dijalankan ke atas 24 pesakit. Ekspresi CD63 telah ditentukan dengan Ujian Pengaktifan Basofil (BAT) dengan menggunakan Penisilin G, Penisilin V, Penentu Utama (PPL), Campuran Penentu Kecil (MDM), Ampisilin, dan Amoksisilin, manakala kuantifikasi spesifik IgE telah dijalankan dengan kaedah Pendarfluor Enzim Imunoasai (FEIA) yang menggunakan reagen Penisilin G, Penisilin V, Ampisilin dan Amoksisilin ke atas semua peserta kajian. Daripada 24 pesakit, seorang pesakit adalah SPT-positif terhadap Amoksisilin dan Ampisilin yang disediakan di makmal dalaman. Dua pesakit adalah BAT-positif dan empat pesakit adalah FEIA-positif. Seorang pesakit menunjukkan keputusan yang konsisten di kedua-dua ujian BAT dan SPT, manakala

seorang lagi pesakit menunjukkan keputusan yang konsisten di kedua-dua ujian BAT dan FEIA. Pemerhatian menunjukkan persetujuan yang agak rendah di antara BAT dan FEIA (Indeks Kohen Kappa=0.25). Walaupun persetujuan ekspresi CD63 di antara BAT dan FEIA adalah agak rendah, nilai diagnostik mereka adalah saling melengkapi. Selain spesifiksiti yang tinggi, kedua-dua ujian menunjukkan sensitiviti yang rendah. BAT adalah berguna terutamanya di kalangan pesakit yang mempunyai sejarah klinikal anafilaksis tetapi negatif kepada ujian FEIA, justeru dapat mengelakkan ujian provokasi ubat yang berisiko tinggi.

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LIST OF SYMBOLS AND ABBREVIATIONS

&	And
β	Beta
Ca^{2+}	Calcium
°	Degree
°C	Degree Celsius
/	Divide by
=	Equal to
≥	Equal to and greater than
γ	Gamma
>	Greater than
Kg	Kilogram
L	Liter
µg	Microgram
µl	Microliter
mg	Milligram
ml	Milliliter
mm	Millimeter
mmHg	Millimeter of mercury
Min	Minute/s
-	Minus
M	Molar
%	Percentage
+	Plus
pH	Potential of hydrogen
ACE	Angiotensin-converting-enzyme inhibitor
ADRs	Adverse drug reactions
Amox	Amoxicillin
Amp	Ampicillin
APC	Antigen presenting cell
BAT	Basophil Activation Test

BCR	B cell receptor
BP	Blood pressure
CCR3	C-C chemokine receptor type 3
CD	Clusters of differentiation
Clav A	Clavulanic acid
DPT	Drug provocation test
DRESS	Drug reaction with eosinophilia and systemic symptoms
e.g.	Example
EAACI	European Academy of Allergy and Clinical Immunology
EAACI-NPS	European Academy of Allergy and Clinical Immunology-Nomenclature Position Statement
EDTA	Ethylene-diamine tetra acetic acid
ENDA	European Network for Drug Allergy
etc.	Et cetera
FcεRI	Fc region of immunoglobulin E
FEIA	N-Formylmethionyl-leucyl-phenylalanine
FITC	Fluorescein isothiocyanate
fMLP	N-Formylmethionyl-leucyl-phenylalanine
HSA	Human Serum Albumin
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
i.e	That is
IL	Interleukin
IV	Intravenous
LAMP	Lysosomal-associated membrane glycoprotein
LR	Lower right
Lys	Lysine
MDM	Minor determinant mixture
MHC	Major histocompatibility complex
p38MAPK	P38 mitogen-activated protein kinases
PAF	Platelet-activating factor

PE	Phycoerythrin
PEF	Peak expiratory flow
Pen G	Penicillin G
Pen V	Penicillin V
PPL	Penicilloyl-polylysine
RAST	Radioallergosorbent test
SI	Stimulation index
sIgE	Specific Immunoglobulin E
SJS	Steven-Johnson syndrome
SPT	Skin Prick Test
SSC	Side scatter
TEN	Toxic epidermal necrolysis
TCR	T cell receptor
Th	T-helper
TM	Trademark
UK	United Kingdom
UR	Upper right
USA	United States of America
WAO	World Allergy Organization

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Medications are fundamental to current healthcare services. However, adverse reaction due to drugs has become a major cause in medical injury especially in susceptible patients. Of all hospital admissions, adverse drug reactions (ADRs) take account for 3 to 6% and responsible for 10 to 15% occurrence in hospitalized patient (Johansson *et al*., 2004). Based on United States of America (USA) National Mortality Database, fatal drug-induced anaphylaxis shows significant increase ($P < 0.001$) from 0.27 per million from 1999 to 2001 to 0.51 per million from 2008 to 2010. Twelve years study in USA revealed 1446 anaphylaxis-related deaths attributable to medications, comprising 58.8% of the anaphylaxis-related deaths in the country (Jerschow *et al*., 2014). Children in general were often to be wrongly diagnosed as being "allergic" to various medications, particularly antibiotics, and bearing the label into adulthood. They are usually prescribed with alternative medication that potentially more toxic, less effective and more expensive, thus resulting an increase of morbidity, mortality and cost (Thong & Tan, 2011).

Beta-lactam antibiotics are widely prescribed for infection diseases. Along with cephalosporins, penicillins are the beta-lactam antibiotics that triggered allergy reactions most frequently with the prevalence of 5% in adults and 10% in children (Romano & Caubet, 2014). A study in Spanish population documented that 9% (66 of 732) of the patients‟ consultations for possible drug allergy were diagnosed to have drug allergy against beta-lactam antibiotics (Gamboa, 2009). Incidence of true penicillin allergy is rare with the estimated frequency of anaphylaxis at 1-5 per 10000 cases of penicillin therapy. However, it was also demonstrated that re-administration of the drug can cause allergic event in up to 60% of penicillin allergic patients (Bhattacharya, 2010).

In allergic reaction, the entire complex of beta-lactam structure bound to carrier protein were recognized and targeted by the immune system. The beta-lactam forms the core chemical structure of penicillin as shown in Figure 1.1. The time interval between last drug administration and symptom(s) onset play an important role to determine if the reaction occur *via* immunoglobulin E (IgE)-mediated immediate type reactions or non-immune mediated allergic reaction. Immediate reactions to beta-lactam are mediated by antigen presenting cells (APCs) through presentation of the entire drug-carrier protein complex to T cells that lead to the generation of T-helper 2 (Th2) cells response, and the production of drug specific IgE (sIgE) antibodies. Cross-linking of these antibodies activate mast cells and basophils to release inflammatory mediators for further recruitment of inflammatory cells are leading to the generation of typical symptoms of IgE-mediated reactions (Chang *et al*., 2012). IgE-mediated reactions that occur within first hour upon drug administration are clinically manifested as urticaria, angioedema, conjunctivitis, rhinitis, bronchospasm, gastrointestinal symptoms, and anaphylactic shock. Patients treated with penicillin are subjected to anaphylaxis risk of 0.002% that could be fatal (Idsoe *et al*., 1968; Saxon *et al*., 1987). Up to 20% of drug-related anaphylaxis deaths in Europe and 75% of deaths for all drug-related anaphylaxis in the USA, are caused by penicillin (Delage & Irey, 1972; Lenler-Petersen *et al*., 1995; Neugut *et al*., 2001).

It is unclear why some people develop penicillin allergies, while others do not. Evaluation of allergy reactions to antibiotics is clinically complex. Allergy diagnostic testings are selected based on detailed clinical features, including symptoms, time interval between last drug administration and symptom(s) onset and reaction type, immediate or non-immediate. IgE-mediated immediate reactions to beta-lactams can be assessed by using several determinants both *in vivo* (such as skin test and/or drug provocation test (DPT)) and *in vitro* testing (blood testing) involving the suspected drug

causing allergic reactions. The traditional test method is the Skin Prick Test (SPT), which indirectly measures sIgE antibodies that bound to the skin mast cells (Oppenheimer & Nelson, 2006). This mode of diagnosis has been widely adopted for the diagnosis of allergic disease. Nevertheless, it should be noted that beta-lactam skin test does not predicts future development of IgE-mediated reactions but predicts only the presence of IgE antibodies for the major or minor penicillin determinants at the time of application. Also, not all penicillin determinants are available commercially for skin testing. In addition, the positivity of SPT may be affected in patients with rash or dermographism (Liccardi *et al*., 2006).

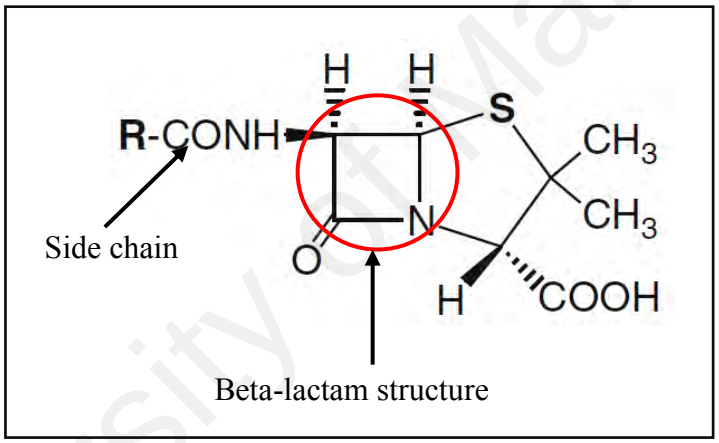

Figure 1.1: Chemical structure of penicillin with beta-lactam structure

Alternatively, the safer method to diagnose allergic reactions to beta-lactam antibiotics is the blood test using Fluorescence Enzyme Immunoassay (FEIA) for the determination of sIgE antibodies (Hamilton & Adkinson, 2004). Immune system of patient with allergic disease produces sIgE antibodies against the drug molecular structure as a defense mechanism. Normal people do not produce the immune reaction to these drugs. On the other hand, the allergy suffers tend to produce abnormally large amount of IgE antibody to these drugs and whom can be measured by FEIA. This method measures both functional IgE (capable to activate mast cells and basophils by

binding to Fc region of immunoglobulin E I (FcεRI) on the cell surface) and non-functional IgE (Khan *et al.*, 2012). However, the sensitivity of FEIA is somewhat low, which varied from 0 to 25% and the specificity varied from 85.7 to 100% (Fontaine *et al*., 2007). In most patients with beta-lactam allergy, the serum level of sIgE antibodies can be declined rapidly, the test often becoming negative within six months to three years after last exposure (Hjortlund *et al*., 2014).

Recently, the development of flow-cytometric technology enables the investigation of the expression of activation markers such as CD63 on the membrane of activated basophils. It has opened a new perspective to diagnose IgE-mediated immediate reactions to beta-lactam. It has been reported from a study in Spain population, that the sensitivity of this test is approximately 50% with a specificity of approximately 93% (Sanz *et al*., 2002).

Basophil Activation Test (BAT) is a cellular-based assay which resembles the *in vivo* pathways that lead to the symptoms, thus making it useful to study the cross-reactivity in quantitative evaluation of residual allergenicity (Mayorga *et al*., 2010). Briefly, the basophils activation pathways are referred as the cross-linking of membrane-bound IgE *via* FcεRI which lead to signal transduction *via* phosphorylation of p38 mitogen-activated protein kinases (p38MAPK) and calcium influx followed by changes on the cell membrane with final degranulation of the basophils. Degranulation of intracellular granules and fusion with cell membrane leads to an expression of CD63 detectable marker on the cell surface. This pathway is shown to be similar with mast cells. But in contrast with tissue-resident mast cells, basophils are easily accessible in the peripheral blood and therefore are favored for experimental approaches for future routine diagnostics (Hausmann *et al*., 2009). This test also relevant for allergic disease diagnosis as compared to SPT because an allergic inflammation might be mediated by basophils independently of mast cells.

A comparative study of FEIA and BAT assays for aeroallergens shows that both tests could distinguish an allergic from an non-allergic person, however, BAT appear inferior to FEIA in distinguishing between allergens to which an atopic person is allergic and same atopic person is not allergic (Khan *et al*., 2012). However, this is only true for aeroallergens and need to be investigated in other allergy causative agents. Moreover, BAT could provide more relevant result compare to FEIA as only functional IgE is measured. The development of advance technologies such as FEIA and flow cytometry (BAT) allows the determination and evaluation of sIgE antibodies using the blood samples. However, the sensitivity and specificity of both assays have not been studied in our clinical diagnostic laboratory. Thus, it would be useful to determine the diagnostic performance of these assays compare to skin testing and eventually to establish a method to facilitate the diagnosis of beta-lactam and/or other drugs allergies in our clinical setting. The general procedures in the present study are outline in Figure 1.2.

Aim of study and objectives

Aim of study

To study the expression of CD63 marker on basophil cells in patients with beta-lactam allergy.

Specific Objectives

- To determine the level of CD63 expression in the activated basophil cells in patients with beta-lactam allergy by flow-cytometry method
- To compare the serum level of specific IgE antibodies and CD63 expression in patients with beta-lactam allergy.

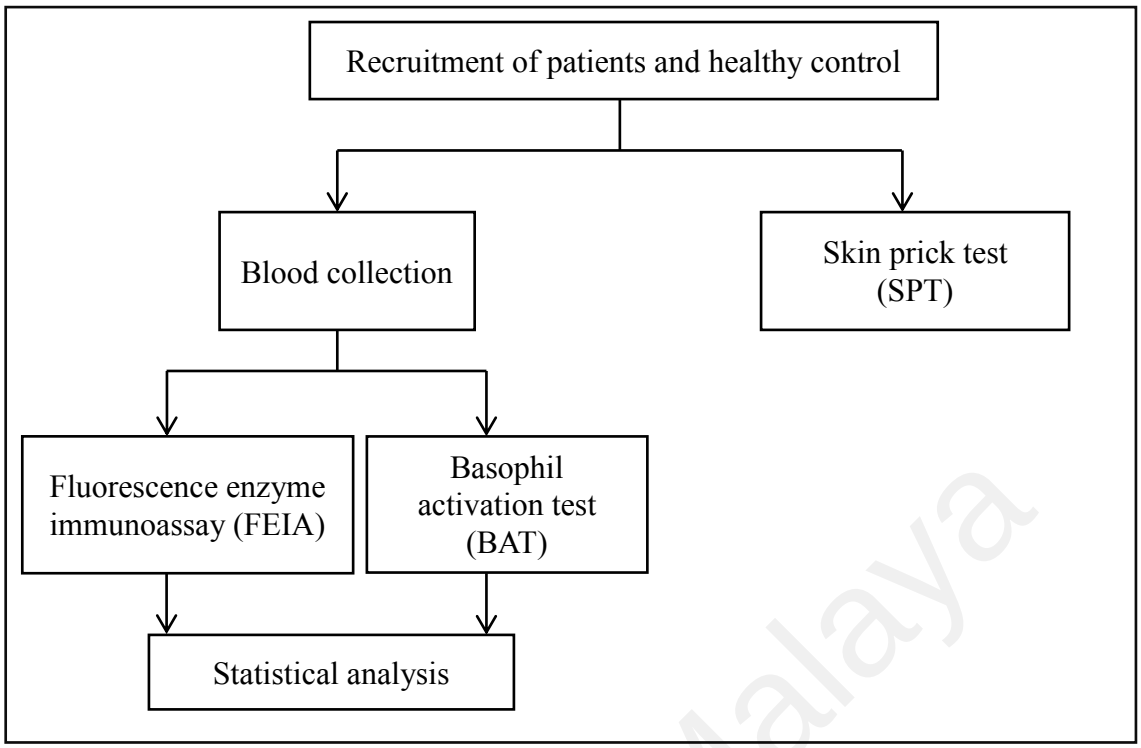

Figure 1.2: Outline of general procedures

CHAPTER 2: LITERATURE REVIEW

2.1 Drug hypersensitivity and drug allergy

Drug adverse reactions are broadly classified into predictable and unpredictable reactions (Thong & Vervloet, 2007). Reactions are varying from severe to even life-threatening, requiring the replacement of prescriptions or discontinuation of favored medications. Undesirable clinical responses in patient under treatment create extra burdens for both patients and doctors. The European Academy of Allergology and Clinical Immunology (EAACI) published an EAACI Position Statement in 2001 entitled, in part, "A revised nomenclature for allergy" (Johansson *et al*., 2001). Upon setting up a Nomenclature Review Committee to review the EAACI position statement, the World Allergy Organization (WAO) set about to promote the described acceptable nomenclature for allergic diseases aiming to improve communication in allergy field globally. As the nomenclature proposed by EAACI was based on reaction mechanisms resulting the signs and symptoms of allergic disease and these mechanisms were usually inflammatory, the WAO Nomenclature Review Committee issued a revised nomenclature for allergy in 2003 to update the nomenclature published in 2001. The revision of nomenclature proposed in The European Academy of Allergology and Clinical Immunology-Nomenclature Position Statement (EAACI-NPS) based on the mechanism initiating the reaction, which is usually inflammatory lead to the signs and symptoms of the allergic disease. Considering that similar inflammation and clinical manifestations can be initiated by different mechanisms, it is important for the researcher, physician, and patient to understand the initiating mechanism. Negligence on that matter may cause inaccurate clinical decisions, inappropriate advice on prevention, and ineffective treatment.

Based on the revised nomenclature, "drug hypersensitivity" defined as reproducible signs and symptoms due to drug exposure at a dose usually tolerated by healthy persons. "Drug allergy" refers to drug hypersensitivity reactions mediated by immune system. These hypersensitivity reactions are classified as immediate reaction (immunoglobulin E (IgE)-mediated) or delayed reaction (non-IgE mediated). Patients with drug allergic symptoms usually develop allergic reactions in the airways involving mucosal membranes down to gastrointestinal tract where the antibody belongs to the IgE isotype. This is specific IgE reactivity to a specific allergen. Allergy cannot be defined on the basis of increase in total IgE level or on the basis of the amount of IgE bound to the cell surface due to unknown allergy-related biological activity of non-antibody active IgE molecules. It has been speculated that in a chronic stage of allergic reaction, the originally IgE-initiated is further dominated by allergen-specific lymphocytes. Other non-immunological factors such as infection, irritants, and exercise, due to hyperreactivity induced by allergic symptoms may also induce and aggravate allergic symptoms (Johansson *et al*., 2004).

The most commonly reported drug allergy reactions involve beta-lactam and non beta-lactam antibiotics. These reactions are clinically manifested by urticaria and/or angioedema, rhinitis, bronchospasm, and anaphylactic shock that develop within one hour after last drug administration (Romano & Caubet, 2014).

2.1.1 Mechanism and clinical manifestations

In IgE-mediated drug reactions, IgE cross-linking, cells activation and released of preformed and newly formed mediators are resulted from binding of drug allergens to IgE antibodies that attached on mast cells and basophils (Baldo & Pham, 2013). The roles of IgE antibodies in both immediate and non-immediate allergic response involving inflammatory reactions are well studied. Binding of IgE antibodies to both its

high- affinity receptor Fc region of immunoglobulin E I (FcεRI) on mast cells and basophils and its low-affinity IgE receptor Fc region of immunoglobulin E II (FcεRII) or CD23 lead to allergic inflammatory reactions augmented by humoral and cellular immune responses (Galli & Tsai, 2012).

2.1.1.1 IgE antibody production

IgE-producing plasma cells usually occur on allergic inflammation site, such as in mucosal, cutaneous, as well as gut lymphoid tissue. Production of IgE antibody start when antigen-presenting cells (APC) bearing specific antigen interact with lymphocytes. APCs are primarily dendritic cells, playing a key role in initiating the adaptive immune response, the macrophages, and B cells. On the other hand, antigen presentations for activation of naïve T lymphocytes are more sophisticated and requiring precise signals. These requirements are meet in two steps *via* APC, firstly by interaction of membrane-associated major histocompatibility complex (MHC) with the T cell receptor (TCR) (activation signal 1), and secondly by the action of co-stimulatory signals in membrane protein ligand CD80 (B7-1) that reacts simultaneously with another membrane ligand CD86 (B7-2) (activation signal 2). Such interaction between ligands to their complementary receptor CD28 is mainly expressed on naïve T cells that leads to cell clonal expansion. Non-dividing naïve B cells also will proliferate and differentiate to effector B cells that will then produce specific IgE (sIgE) antibodies. B cells producing these antibodies required the participation of B cell receptor (BCR) and co-stimulation from T helper cells. The activation of B cell is initiated when there is interaction between complementary antigen and immunoglobulin anchored on the BCR in the cell membrane. The BCR-antigen complex is internalized within an endosome, processed and presented to the surface by MHC type II molecules to T cells. Presentation of the antigen to Th2 cells lead to the production of IL-4 and IL-13 cytokines which then promote B cell proliferation and isotype switching for IgE

antibody production. Density of IgE receptor on cell surface is influenced by IgE levels. Increase level of the antibody will increase FcεRI receptor density that will promote mast cells and basophils degranulation. Following degranulation, increase release of cytokines from Th2 cells will further stimulate IgE production and increases receptor density. Similarly, decrease of IgE levels leads to decrease of mast cells receptor and thus reduce degranulation (Gould *et al*., 2003).

2.1.1.2 Mast cells degranulation release inflammatory mediators

The role of IgE antibody in both immediate and delayed allergic is well studied. The resultant humoral and cellular interactions along with mast cell, produces various inflammatory mediators are leadingto the development of allergic symptoms. Interactions of IgE antibodies with high-affinity IgE receptor (FcεRI) that mainly expressed on the surface of mast cells and basophils initiated the cells activation causing the released of mediators. High affinity of the receptors indicate IgE antibodies are bound in high proportion even when there are low circulating antibodies.

As occurs in anaphylaxis, the most important event, initiating the release of mediators is the cross-linking of receptor-bound IgE by drug allergen molecules on the mast cells. The IgE-FcεRI formation is long-lasting and dissociation is very slow. Receptors cross linking lead to their aggregation, activation protein tyrosine kinase (Lyn and Fyn), and finally transphosphorylation of the β and γ chains with Syk kinase involvement. The phosphorylation reactions induce a series of activation steps that occur within seconds lead to mast cell degranulation. Several inflammatory mediators released during mast cells degranulation are preformed or newly synthesized and stored in the cytoplasmic granules. These which includes histamine, heparin, platelet-activating factor (PAF), serotonin, enzyme such as tryptase, chymase, and carboxypeptidase, and eosinophil, neutrophil, and monocyte chemotactic factors. The released of preformed mediators from mast cells granules cause immediate allergic

reactions symptoms such as, itching, bronchoconstriction, vasodilation and edema. Histamine, cysteinyl leukotrienes, and PAF are among the important mediators in type I immediate allergic response (Baldo *et al*., 1991; Brown *et al*., 2008; Gilfillan & Tkaczyk, 2006).

2.1.1.3 Amplification of IgE antibody production

Production of IgE antibodies by the B cell is accentuated by mast cells, basophils, and even dendritic cells by direct interaction. Newly synthesized IgE antibodies bind to high affinity FcεRI receptors on the surfaces of mast cells and basophils. Activation of receptors, resulted from IgE cross-linking triggers the cells to express CD40 ligand (CD40L) and secrete IL-4. The IL-4 will then react with their complementary receptors expressed on the surface of B cell, mast cells and basophils which induce isotype switching and increase the production of IgE antibody (Janeway *et al*., 2001).

2.2 Risk factors of drug allergy

The risk factors in most of drug allergy patients are vaguely defined and not well identified. Anticipating a drug reaction usually involves little certainty excluding well studied drug, for example Carbamazepine and Allopurinol. In most cases, considering the obvious risk factors linked to the drug and the patient can be useful. The association between drug administration and rashes are usually temporary especially when beta-lactams drugs like Ampicillin and Amoxicillin are administered in children. However, in most cases, it seems likely that allergic reaction is the results of infectious agent (e.g., in infectious mononucleosis) or the interaction between the infectious agent and the drug. Such responses, although show similar clinical manifestations do not appear to be immunologically mediated (Thong & Tan, 2011).

Higher sensitization rate to drugs was not shown by atopic patients; however, they are at higher risk of having severe reaction once it occurs. This is also true for patients with uncontrolled allergic asthma and food allergies. There are few studies (Bharadwaj *et al*., 2012; Daly, 2012; Wilke *et al.*, 2007) indicate the importance of genetics and ethnicity in certain drug allergies where the situation has further complicated the pathway by the involvement of multiple genes as well as environmental factors. Investigation of the associations between drug allergies and human leukocyte antigens (HLA) of the MHC on chromosome 6 remains a continuous area of research. Further discoveries of the relationship between genetics and drug reactions will help to identify patient in risk prior the administration of a potentially harmful drug.

Repeated exposure to drug also will increase sensitivity and one should always need to be cautious of cross-reactivity with other medications. Examination of drug-related risks includes the first and most obvious consideration, which is the nature of the drug. It is important to consider the chemical properties of the drugs, particularly the molecular weight, structural complexity and chemical reactivity. Drug dosage, the duration and frequency of administration may also affect the reaction risk because a prolonged administration of high dosage can increase the risk to develop drug allergic reaction (Talbot *et al*., 2012).

Route of drug administration have significantly contributed to high incidence of anaphylaxis due to drug given intravenously. Intramuscular administration carries slightly higher risk than subcutaneous followed by oral route being the safest mode of administration. Nevertheless, at lower risk, oral administration still can cause sensitization and severe reactions (Baldo *et al*., 2011). Drugs associated with high incidence of sensitization, such as antibiotics chloramphenicol, penicillin, neomycin and sulfonamides that are frequently administered by topical application should be avoided. Finally, specifically in beta-lactam antibiotics, due to general cross-reactivity on

structural basis, one should have basic knowledge of the chemical structures of the agents prior to prescribing the drugs or change of medications (Edwards & Aronson, 2000).

2.3 Beta-lactam allergy

Antibiotics are classified as beta-lactam and non beta-lactam. Beta-lactam consists of four main classes which include penicillins, cephalosporins, monobactams, and carbapenems, all of which contain a four-membered beta-lactam ring as shown in Figure 2.1. Non beta-lactam antibiotics (e.g. quinolones, macrolides, sulfonamides, glycopeptides, clindamycin, aminoglycosides, and rifamycins) have distinguished chemical structure, antimicrobial property, and immunogenic capability. The prevalence of hypersensitivity reactions to antibiotics are approximately 10% and commonly reported both in adults and children (Solensky, 2012).

Beta-lactam antibiotics are the most reported drug related to drug allergic reactions mediated by specific immunological mechanisms. All currently available beta-lactams, ranging from benzylpenicillin to more recently introduced beta-lactams, such as aztreonam or the related beta-lactamase-inhibitor clavulanic acid are capable to induce allergic reactions (Torres *et al.,* 2003).

2.3.1 Prevalence and epidemiology

Approximately 10 to 20% of the general populations have been labeled as penicillin allergic (Macy & Ngor, 2013). Penicillin is the most common beta-lactam antibiotic allergy and the most common drug class of allergy, reported in about 8% of individuals using health care system in the USA. It is the most widely used antibiotic families in the USA, with Amoxicillin or Amoxicillin combination products accounting for the vast majority of the courses (Macy, 2014). Penicillin also known to induce drug allergic reactions responsible for an estimated 75% (500-1000) of deaths each year in

the USA and 26% of fatal drug-induce anaphylaxis in the United Kingdom (UK) (Miles & Bain, 1992; Neugut *et al*., 2001). However, most reported penicillin allergy is not associated with clinically significant IgE-mediated reactions after penicillin re-challenge (Macy, 2014). Studies also reported that more than 90% of the patients labeled with allergic are able to tolerate penicillin upon assessment (Borch *et al*., 2006; Lee *et al*., 2000).

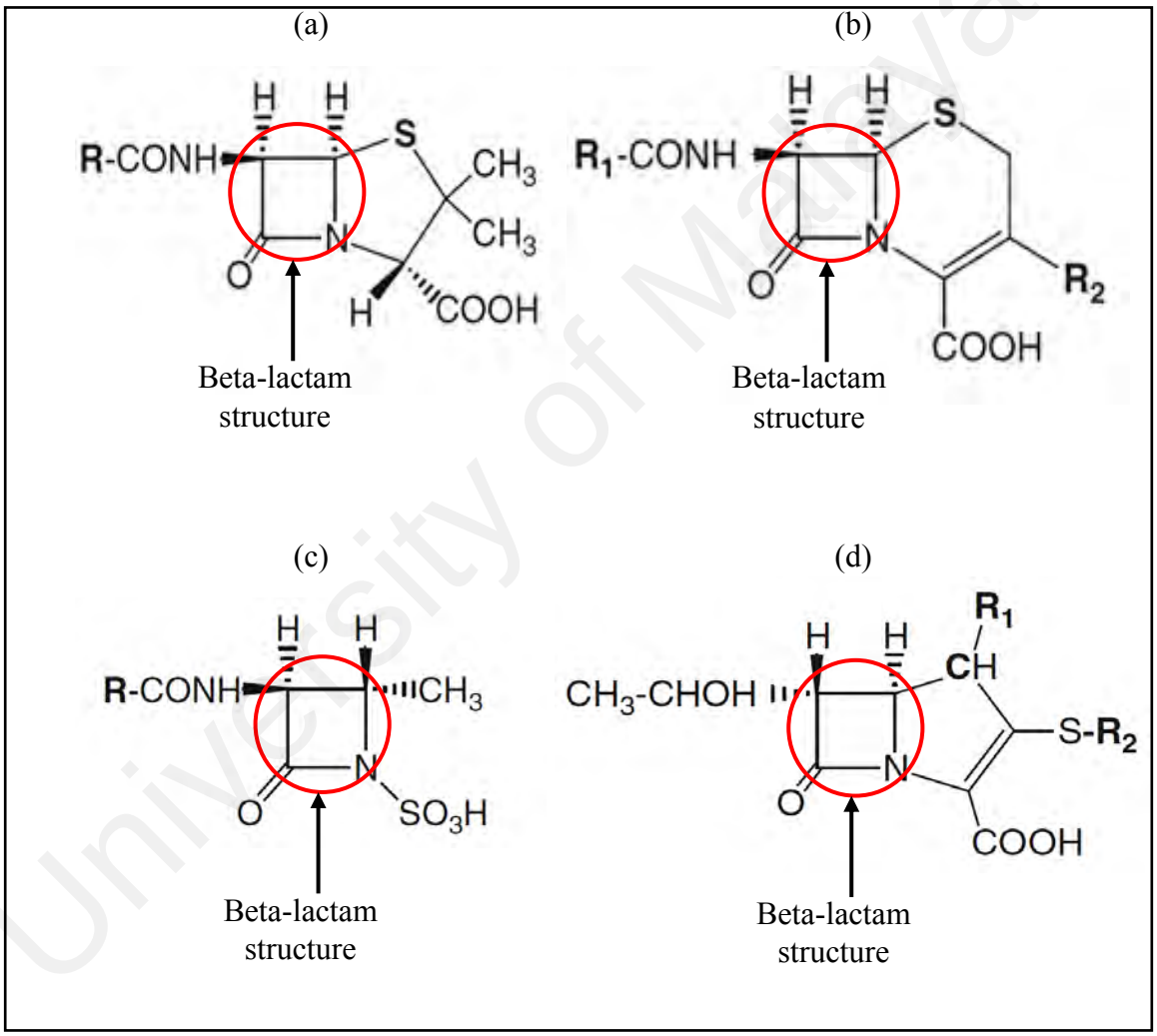

Figure 2.1: Chemical structure of (a) penicillin, (b) cephalosporin, (c) monobactam and (d) carbapenems with beta-lactam structure.

2.3.2 Beta-lactam antibiotic

The beta-lactam antibiotics are categorized into four main drug classes that have antibacterial properties; penams (penicillins), cephems (cephalosporins), monobactams, and carbapenems. The beta-lactam ring is fused to a thiazolidine ring in penams, a dihydrothiazine ring in cephems, and a dihydropyrrole ring in carbapenems. Monobactams consists of a beta-lactam ring free of any other ring attachment (Nathwani & Wood, 1993).

2.3.2.1 Penicillin

The formation of antigenic and allergenic determinants of benzylpenicillin (Penicillin G) has unraveled the intricate pathways and steps in the formation of allergenic determinants. Extensive study shows that, penicilloyl is the most significant determinant of all the penicillin breakdown products and protein conjugates. Penicilloyl determinant has been generally agreed as the major determinant in penicillin allergy (Batchelor *et al*., 1965).

The designation of the major penicillin antigen was driven primarily from the major populations of antibodies in sera from experimental animals immunized with benzylpenicillin, and from humans following penicillin therapy, which found to be complementary to penicilloyl determinant (Baldo *et al*., 1995; Liakopoulou & Perelmutter, 1975). Penicilloyl groups are estimated to be approximately 95% from all penicillin molecules that covalently bound to protein under physiological conditions. This quantitative predominance leads to the application of the term „major" rather than allergenic potency. Formation of penicilloyl determinant occurs in more than one pathway. One is by direct reaction of benzylpenicillin involving the opening of the beta-lactam ring and nucleophilic attack on protein amino group at high basic pH. Study also showed that the reaction can occur in neutral pH (Schneider & De Weck, 1967). Benzylpenicillin are readily rearranged to form an isomer, D-benzylpenicillenic acid, a

highly reactive compound which like benzylpenicillin, exclusively bind to lysine residues of human serum albumin (HSA) forming penicilloyl-lysine adducts. Benzylpenicillin and benzylpenicillenic were shown *in vitro* to covalently bound to lysine residues in HSA to form penicilloyl adduct (Meng *et al*., 2011). However, in their study, benzylpenicillin showed marked preferential binding to Lys 199 and benzylpenicillenic acid bound to Lys 199 as well as Lys 525.

Benzylpenicillic acid, which forms readily from benzylpenicillin in aqueous solution, is unstable and is thought to be allergenic. Immunization laboratory animal with penicillenate-protein and penicilloyl-protein conjugates revealed that the penicillenate and penicilloyl haptens were identified as different determinants. Although penicillenate determinant is not a clinically important allergen, detection of complementary IgE antibodies against the compound has shown its‟ allergenic properties (Singh *et al*., 2004).

Hydrolysis of benzylpenicillin produces benzylpenicilloic acid as the main product that capable to elicit wheal and cause skin eruption in some patients. It is considered to be one of the minor determinants. Additionally, penilloic acid, which is the decarboxylation product of penicilloic acid, is another minor determinant. It has been suggested that in vivo, penicilloic acid reacts with cysteine disulfide linkages via its penalmadic acid intermediate to form benzylpenalmadic acid cysteine mixed disulfide and, then, via a penamaldate rearrangement, to penicillamine cysteine mixed disulfide and benzylpenilloaldehyde (Levine & Redmond, 1969). Penicilloic acid degradation products can form in vivo for it is chemically capable to react with protein carriers and form a complex that can function as allergens. However, data of the allergenic properties of this determinant is scanty.

Other than the penicilloyl moiety, metabolites are believed to constitute about 5% or less of administered penicillin, which often referred as minor determinants (Greenberger, 2006). Levine *et al.* (1969), in their study concluded that "immediate allergic reactions to penicillin is the most often mediated by skin-sensitizing antibodies of minor determinant specificities", while penicilloyl-specific skin-sensitizing antibodies are "invariably associated with accelerated and late urticarial reactions and probably mediate these reactions". Immunoglobulin G (IgG) and Immunoglobulin M (IgM) were thought to be the main penicilloyl-specific antibodies and these antibodies act as blocking antibodies to prevent penicilloyl-mediated immediate reactions. It was originally recommended that the diagnosis of penicillin allergy should be using two skin test solutions-benzylpenicilloyl-polylysine conjugate (PPL) at a concentration of 10^{-6}M and a minor determinant mixture (MDM) consisting of potassium benzylpenicillin, sodium benzylpenicilloate, and sodium benzylpenilloate, all at a concentration of 10^{-2}M. The PPL contained 20 lysine residues with 13 of them coupled to the penicilloyl hapten and the remaining lysines succinylated. Skin tests on penicillin-allergic patients, showed heterogenous responses with some reacted only to benzylpenicillin, only to penicilloate, or only to PPL or any combination of the test reagents. Hence, to prevent misdiagnosis in penicillin-allergic individuals, penicillin, penicilloate, penilloate, penicilloyl-amine, and PPL must be used for skin testing (Levine *et al*., 1966).

2.3.2.2 Cephalosporins

Cephalosporins are classified based on the development sequence and an antimicrobial mechanism of action known as first or subsequent (second, third, and so on) generation beta-lactams. Cephalosporin beta-lactam are the most widely used beta-lactam antibiotics after penicillin to treat common infections. Cephalosporins and penicillin share a common beta-lactam ring, meaning the two groups might strongly

cross-react to display similar antigenic and allergenic properties. The incidence of allergic reactions to cephalosporins is between 1 to 3% (Campagna *et al*., 2012; Romano *et al*., 2004) with skin reactions, primarily rashes, urticaria, and pruritus occur at about 1 to 3% and anaphylaxis up to 0.1% (Pegler & Healy, 2007).

Nevertheless, this evidence may be underestimated since there is no large surveys seem to have been undertaken allergic reactions to cephalosporins. It was found to be 9.2% in patients with history of penicillin allergy (Sodhi *et al*., 2004), while 1.7% patients developed reaction despite negative history to penicillin (Gadde *et al*., 1993). Thus, patients with a history of penicillin allergy are 5.4 times more likely to have allergic reactions to cephalosporins.

2.3.2.3 Monobactams

Monobactams is a monocyclic drug containing only the ring structure of beta-lactam antibiotics. Aztreonam is monobactam synthetic compound containing a thiazolyl group in the side chain and sulfate group attached to the nitrogen of the beta-lactam ring. Aztreonam has been shown minimal to no cross-reactivity in the initial study assessing cross-reactivity between the drug and other beta-lactam antibiotics. Such findings lead to the prediction that the side chain could be the main immunogenic side of the drug instead of the beta-lactam nucleus. This prediction was confirmed when ceftazidime, a cephalosporin with a side chain identical to aztreonam, completely inhibited by the rabbit anti-aztreonyl antibodies (Adkinson *et al*., 1984). The lack of cross-reactivity with penicillin determinants was also demonstrated in the failure of aztreonam-protein conjugates and free drug to react with human anti-benzylpenicilloyl IgE antibodies (Adkinson *et al*., 1985).

Nevertheless, IgE antibody-mediated reactions including urticaria, angioedema, and anaphylaxis on first exposure to aztreonam still occur albeit being well tolerated in patients allergic to beta-lactam antibiotics. Caution should be taken when prescribing aztreonam to cystic fibrosis patient with history to other beta-lactam antibiotics due to the possibility of allergic reaction on repeated usage. Cross-reactivity assessment between aztreonam and other beta-lactams in cystic fibrosis patients with negative history demonstrated two patients developing anaphylactic reactions to the drug (Moss, 1991). Another retrospective study of allergic reactions to aztreonam and other beta-lactams in patients with cystic fibrosis showed frequent reactions ranging from 50.9% for piperacillin to 4% and 6.5% to imipenem and aztreonam, respectively. Seemingly, cross-reactivity and the reactions to aztreonam are restricted to particular group with high risk of allergy to beta-lactams (Parmar & Nasser, 2005).

2.3.2.4 Carbapenems

Carbapenem nucleus contains an unsaturated five-membered ring as for penicillins but with the sulfur atom of the latter replaced by a carbon atom. Imipenem and meropenem are the most prescribed drugs from this group. The frequency of cross-reactivity between imipenem and penicillins as defined by skin testing was about 47% (Neftel & Cerny, 1992). Administration of imipenem to patient with high risk of penicillin allergy should be regarded with caution because imipenem can cross-react with penicillin especially in minor determinant form. In the general population, the frequency of carbapenems allergy has been estimated to less than 3% (Calandra *et al*., 1988).

2.3.2.5 Clavams

Clavams (clavulanic acid) is a bicyclic structure with a beta-lactam ring but lacking a R1 side chain with an oxazolidine ring as shown in Figure 2.2. Clavams has non-significant antimicrobial activity but binds to the active site of beta-lactamase enzyme and inhibits the function. The compound is usually formulated with penicillins such as amoxicillin and ticarcillin to retain its antimicrobial activity and to prevent inactivation. It was demonstrated that the compound was poorly immunogenic with low to no allergenicity (Salvo *et al*., 2009). Recent increase of allergic reactions to clavulanic acid, reflected the increased usage of the compound with amoxicillin. Furthermore, diagnosis of allergy to clavulanic acid was hampered by the limited availability and stability of the enzyme inhibitor and its presence in combination with antibiotic.

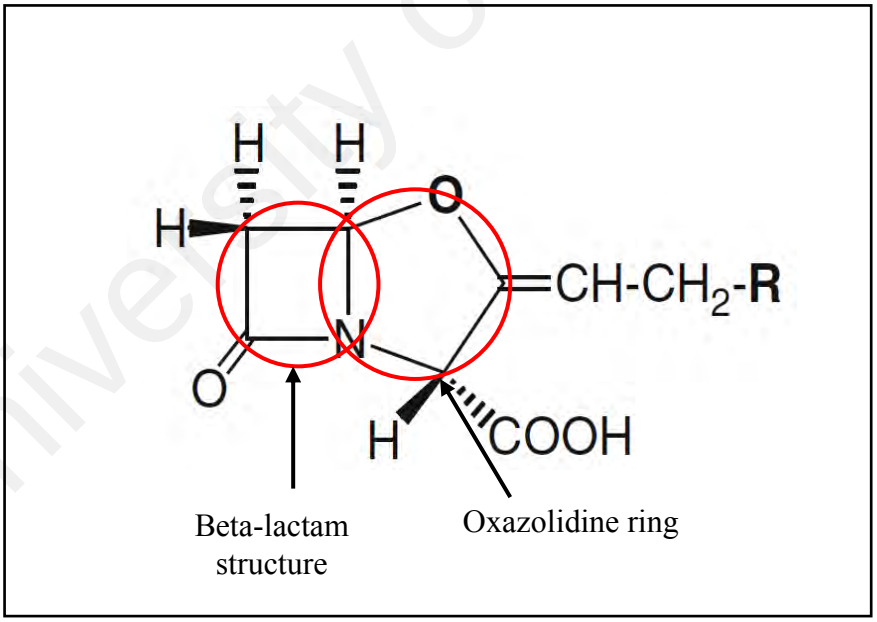

Figure 2.2: Chemical structure of clavams with beta-lactam structure and oxazolidine ring

2.4 Diagnosis of drug allergy

Diagnosis of drug allergy to antibiotics is usually complex. Although guidelines for diagnosis and management for drug allergy have been accessible for many years, clinical approach is diverse across the world affected by different origin of undergraduate and postgraduate allergological training, type of allergological practice, funding mechanisms, accessibility to various types of diagnostic tests, availability of basic versus tertiary practice facilities/laboratory equipment, and many other factors. A study on diagnostic and management in drug allergy involving 82 members of World Allergy Organization (WAO) revealed that 74.7% are using skin test with only 71.4% have access to penicillin skin test reagents. *In vitro*-specific IgE tests were used by 67.4%, and basophil activation test was used by 54.4%. Lymphocyte transformation tests were used by 36.8% and patch tests by 54%. Drug provocation tests (DPT) were used by 68.4% and 76.9% excluding drug allergy based on negative history or symptoms (Thong *et al*., 2011).

Allergy to beta-lactam antibiotic are usually diagnosed based on patient‟s clinical history and positive skin tests, or specific IgE antibody measurements (Blanca *et al.,* 2009). Patient‟s clinical history is crucial because allergic examination is attributed by *in vivo* tests where patients were selected based on clinical features and type of reactions assessed by the outcome of skin tests and/or DPT (Romano & Caubet, 2014). Most patients with history of beta-lactam allergy have no evidence of IgE antibodies to penicillin on the available testing and avoid the antibiotics unnecessarily.

2.4.1 Detail clinical history

Patient‟s clinical history is the most important component in the process of diagnosis. If the clinician spends sufficient time on questioning and analyzing the case history, subsequent assessment or testing might not be necessary.

The symptoms reported by the patient along with the clinical signs confirmed by direct physical examination and/or clinical and laboratory tests, allow the clinician to make accurate diagnosis based on pattern recognition, context, and probability. A series of relevant and specific drug-related questions is necessary when gathering clinical information together with standard patient information. Acquiring the following information aimed at providing answers will help the diagnosis, the treatment if needed, and the formulation of a future avoidance strategy (Baldo & Pham, 2013).

1. List of all the medications the patient is, or has been taking including over-the-counter preparations.
2. The quantity of drugs taken and time duration.
3. Most suspected drugs causing the reactions and why.
4. Time when the reaction begun and duration.
5. Time duration from the initiation of therapy and the symptoms onset.
6. Did the reaction occur on first exposure to a drug?
7. List of all symptoms including description of skin reaction.
8. Recent medical or dental procedures such as major or minor surgery, radiographic investigation, immunization, or tooth filling or extraction.
9. History of drug allergy and previous reaction to suspected drug.
10. Family history of drug allergy and other allergies.
11. Recent viral infection.
12. Other disease, in particular, asthma, cystic fibrosis, diabetes, etc.
13. Questions on home environment, pets, hobbies etc.

A firm diagnosis can be established if questions 3, 4, 5, and 7 are adequately answered. Mechanism of reaction also can be determined if the information on the time of symptoms onset are essentially provided in questions 4 and 5. Immediate IgE

antibody reactions manifested as simple rash to life-threatening anaphylaxis usually occur from a few minutes to one hour after drug administration. In response to question 7, direct view or photograph (e.g., of skin reactions) of the clinical manifestations during the reactions can be very useful. Symptoms resulted from mast cells activation such as anaphylaxis, bronchospasm, angioedema, and urticaria are signs of IgE mediated reactions.

Identification of culprit drug are often challenging if the patients are taking multiple drugs especially in surgery, when several drugs were concurrently administered. Detail investigations are often needed especially for reactions occur during anesthesia. In most of the cases it is the anesthetist or physician who is in charge are responsible to identify the culprit agent and record the vital information is recorded for further reference.

2.4.2 Skin test

Skin testing for beta-lactam allergy has been limited during the past decade because of the lack of commercially available reagents (Macy *et al*., 2010). Skin tests with major (PPL) and minor (MDM) penicillin determinants are considered the first diagnostic procedure in the assessment of immediate allergic reactions. However, a study has described a decrease in the diagnostic sensitivity of these determinants (Torres *et al*., 2003). This could be due to a significant proportion of patients produce specific and selective antibodies against side chain of Amoxicillin or the other beta-lactam antibiotics. There is some controversy about what determinants should be included in the evaluation of an allergic reaction to beta-lactam (Geng *et al*., 2017; Khan & Weiss, 2013).

Identification of penicillin metabolites and breakdown products in the early research has allowed the development of diagnostic testing. In the early development of first major determinant (PPL) in 1961, it was used for skin testing at a maximum concentration of 10^{-6}M and later, the minor determinants, potassium benzylpenicillin, sodium benzylpenicilloate, and sodium benzylpenilloate were each used at a concentration of 10^{-2}M (Al-Ahmad *et al*., 2014). Increase usage of semisynthetic penicillins such as Amoxicillin and Ampicillin increase the incidence of drug allergic reactions which have mark the allergenic importance of the side chain structures. Amoxicillin and Ampicillin are now often included as standard reagents in skin tests. These drugs usually used at maximum test concentration at 20 mg/ml for SPT and intradermal testing. Steady access to testing reagents for penicillin allergy has encouraged diagnostic skin testing to patients with history of penicillin allergy.

Although severe reactions have been reported following skin testing with penicillin reagents, it is considered as a safe procedure with a systemic reaction rate of about 1% or less (Liccardi *et al*., 2006). Nevertheless, potential danger is anticipated especially patients with apparent history. The rate of positive skin test in patients with no history of penicillin is about 2 to 7% and most penicillin-induced anaphylactic deaths occur in patients with no apparent history of a reaction to the drug (Gonzalez-Estrada *et al*., 2015).

Performing SPT is crucial to eliminate the possibility for severe reactions that can occur if higher than recommended test concentrations is used intracutaneously. Skin testing procedure must be done in the facilities with ready access to the appropriate medications and equipment with the presence of physician capable to manage anaphylaxis. Notably, skin tests may appear negative for up to two weeks or longer upon anaphylaxis episode which lead to the important consequences if the culprit drug

was not identified. Retesting is advisable after three to six weeks in patients who were tested negative upon anaphylactic episode (De Weck & Bundgaard, 1983).

Although skin tests using PPL have been positive in up to 70% of patients with immediate type I reactions to penicillin, a testing done on 290 patients with history of immediate allergic reactivity to penicillin (71% anaphylaxis, 29% urticaria) revealed skin test sensitivities of 22% for PPL, 21% for MDM, 43% for Amoxicillin, and 33% for Ampicillin. Positive skin test to at least one determinant occurred in 70% of the patients, indicate that 30% of patients could be misdiagnosed (Torres *et al*., 2001). Therefore, skin testing, based on its sensitivity is a long way from ideal due to unsteady results even with the usage of four different determinants.

Negative skin test was also observed in patients‟ positive drug provocation test. This is contradicting with popular belief that reaction is negligible in this group of patients. Thus, one should be cautious with the possibility of drug reactions to penicillin in subject with negative skin tests to the major and minor determinants. To establish skin test sensitivity and specificity to penicillin, the data from tested tolerance subjects is essential.

Skin test result can be read within 15 to 20 minutes after completing the skin test. Saline is usually used as negative control and histamine chloride as positive control indicate by 3 mm wheal accompanied by erythema. Patients is advised of the possibility of delay reaction. A positive delay reaction to skin testing are manifest as erythema, papulation, infiltrate, eczema, and swelling. Infiltrated erythema with a diameter greater than 5 mm should be considered a positive reaction. Skin test reactivity is generally decreased with time. In a 13 years‟ study (1995 to 2007), there has been a steady decline in the rate of positive skin test declined from greater than 10% to less than 5%. Although, the rate of positive findings is decreasing and is the lowest in older patients, skin test positive still occurs. Thus, skin test is still a very useful clinical tool especially

in older people who are more likely to be hospitalized and more likely to require antibiotics (Macy *et al*., 2009).

2.4.3 Drug provocation test (DPT)

Drug provocation tests (DPT) are noted as "the gold standard" to rule out the presence of drug allergy. However, DPT are restricted by ethical and practical issues thus not convenient for routine diagnostic practice (Ebo, 2011). Diagnostic DPT have mainly been applied for research purposes and have still not entered mainstream clinical practice (Decuyper *et al*., 2016)

In the diagnosis of drug allergy reactions, DPT is a regulated step-wise drug administration in a supervised hospital setting to determine if the suspected drug was the causative agent in a patient‟s allergic reaction (Chiriac & Demoly, 2013). The test is also employed to determine if alternative drug can be safely administered to a patient. DPT is considered as the "gold standard" in the diagnosis of drug allergy reactions and best way to confirm an allergic reaction because of the reproducible clinical signs and symptoms to the original upon testing. Positive provocation test suggests a clear need to avoid the drug allergen while negative results get rid of patient‟s misdiagnosis as allergy to the drug. DPT may be the only reliable way to achieve a diagnosis if skin test or serum IgE antibody results were inconclusive. Upon assessment of low risk of serious allergic reaction, provocation test to the suspected drug can be initiated. However, performance of this testing should take account the decision after careful consideration of risk to benefit ratio.

2.4.4 IgE quantification

Thorough history and skin tests are also complemented with *in vitro* quantification of sIgE antibodies when an IgE-mediated mechanism with the activation of mast cells and basophils is implicated. In certain cases, negative skin test to the suspected drugs in patient with history to immediate reaction require complementary IgE testing for diagnosis confirmation.

Shortly after the development of *in vitro* detection to quantify an allergen specific IgE antibodies known as radioallergosorbent test (RAST), preparation of benzylpenicilloyl and phenoxymethylpenicilloyl protein conjugates immobilized on a solid phase were used to test sera of penicillin-allergic patients. Of eleven patients, nine were found to have reactive IgE antibodies against penicilloyl determinants. Skin test results were in agreement with RAST test results for both positive and negative reactors (Wide & Juhlin, 1971). Several important information acquired in the early applications of penicillin RASTs include the finding that the penicillanyl determinant yielded no more information than the penicilloyl determinant, cross reactivity between penicillin minor determinants and the major determinant, and rare positive reactions to penicillamine. Preparation and examination of thiol-linked penicillamine, benzylpenicillenic acid and the benzylpenicillanyl determinant by Dewdney‟s group is perhaps the most useful information in the early applications of the RAST to diagnose penicillin allergy (Batchelor *et al*., 1965). The discovery of these reagents has essentially confirmed the importance of the penicilloyl determinant, but, most importantly, the study also confirmed that the various immune response to penicillin extending to the specificity of the serum IgE antibodies. The widely distributed Phadia ImmunoCAP® (Thermo Scientific) of drug-solid phases for Penicilloyl G and V, Amoxicilloyl, and Ampicilloyl determinants is the most known commercially available test reagents for penicillin-reactive IgE antibody detection. This assay is capable to

quantify specific IgE antibodies in the range 0.01–100 kUA/l with a cutoff value of 0.35 kUA/l for a positive result and levels higher than 0.1 kUA/l, indicating sensitization to the drug. Assessment of ImmunoCAP® assays (Benzylpenicilloyl and Amoxicilloyl) using sera from positive skin tests patients to benzylpenicillin-derived agents and Amoxicillin revealed sensitivity of 54% with a specificity of 95 to 100%. Testing of Amoxicilloyl ImmunoCAP® assay on 29 sera from patients‟ positive skin test to Amoxicillin but negative to PPL and MDM revealed a sensitivity of 41% and 42% among 26 skin test negative patients (Blanca *et al*., 2001). Patients who are positive provocation test were positive in the immunoassay, indicating the potential to eliminate provocation tests by performing IgE quantification. Immunoassay sensitivities, but not necessarily specificities, for penicillins developed in individual laboratories varied with the commercial assay. In individual laboratories, sensitivities are between 42.9 to 75% and specificities are between 66.7 to 83.3%. As for commercial assay sensitivities are between 12.5 to 24% and specificities are 83.3 to 100% (Fontaine *et al*., 2007). Similar results demonstrated in a comparison study using both penicilloyl and penicillanyl derivatives of benzylpenicillin and Amoxicillin. Detection of benzylpenicillin and Amoxicillin-reactive IgE antibodies in the sera of 28 patients diagnosed with immediate hypersensitivity reactions to a beta-lactam demonstrated sensitivities of 57.1% and 78.6%, respectively, while the corresponding figures for the ImmunoCAP® assays were 35.7% and 28.6%. Nevertheless, specificities of 80.7 to 87.3% for the laboratory tests were less than the results of 86.3 and 98.2% obtained with the commercial assays (Baldo & Pham, 2013). Therefore, some improvement is still needed with regards to sensitivity of IgE quantification to different penicillin.

2.4.5 Basophil Activation Test (BAT)

Unlike tissue-resident mast cells, basophils are easily accessible from the peripheral blood make it an ideal cell indicator for IgE-mediated reactions besides its high-affinity IgE receptors on the cell surface. Cross-linking of drug allergen on membrane-bound IgE antibodies (*via* FcεRI) lead to basophils upregulation activating various surface marker which include CD63 and CD203c. Activation of these marker on basophil surface can be detected using specific monoclonal antibodies *via* flow-cytometry (Hausmann *et al*., 2009).

To date, the application of BAT for diagnosis of beta-lactam allergies have been described in nine studies. Several large-scale studies demonstrated consistent sensitivity at approximately 50% in patients with positive clinical history and skin tests. BAT shown to have better sensitivity with approximately 10% higher compare to commercial sIgE quantification with specificity more than 90%. Thus, this marks the clinical importance of positive BAT results. Importantly, BAT was positive in 25% of patients with positive provocation test and sIgE negative, and in 37% of patients with positive clinical history but negative skin tests (Abuaf *et al*., 2008; De Week *et al*., 2009; Eberlein *et al*., 2010; Gamboa *et al*., 2004; García-Ortega & Marín, 2010; Sanz *et al*., 2002; Torres *et al*., 2010; Torres *et al*., 2004; Torres *et al*., 2011). These results suggest

the importance of BAT as an alternative test when drug allergy is highly suspected but is not supported by results of skin testing or *in vitro* IgE measurements. Because sIgE tests are not available for most cephalosporins, BAT can be developed further for diagnosing allergies to a wider range of beta-lactams.

2.4.6 CD63 expression as basophil activation marker

Basophils are highly granulated leukocytes containing histamines and can release their mediators when activated in response to FcεRI crosslinking by allergen or artificial cross-linkers (Siracusa *et al*., 2013). In basophils activation, the cells undergo rapid morphologic changes and exocytosis of intracellular granules containing preformed mediators. The cells degranulation express unique surface markers such as CD63 which can be measured by flow cytometry (Boumiza *et al*., 2005). CD63 is expressed on the surface of degranulated basophils and is the best-validated activation marker used to quantify basophil activation. CD63 also known as lysosomal-associated membrane glycoprotein-3 (LAMP-3; also known as granulophysin), is a 53 kDa member of the transmembrane-4 superfamily (tetraspanins) (Knol *et al*., 1991).

In resting basophils of both normal and allergic subjects, CD63 is located in the intercellular granules with little surface expression. After stimulation by FcεRI, these granules fuse with the plasma membrane and thus, CD63 is expressed on the membrane surface of degranulated basophils in high density (MacGlashan, 2010). CD63 expression on basophils has produced convincing and specific results with some common inhalant and venom allergens, but with respect to drugs, some early studies reported sensitivities on only 50-64%, that is, not sufficient to be clinically useful. It was suggested that a contributing factor to this poor sensitivity may be the expression of CD63 on other activated leukocytes, including platelets, and the subsequent adhesion of these other cells to basophils (Erdmann *et al*., 2004; Kleine-Tebbe *et al*., 2006). In such case, a more specific and sensitive marker therefore seemed desirable.

Buhring *et al*. (1999), described the monoclonal antibody 97A6 defined a novel surface antigen belonging to the type II transmembrane protein family on human basophils referred to as CD203c. CD203c is constitutively expressed in low levels on basophil surface membrane present on CD34+ progenitor cells and mast cells. Upon

allergen stimulation, CD203c is rapidly upregulated making it a valuable marker for basophil activation and hence allergy diagnosis. Interestingly, it was thought that the release of histamine is not directly associated with expression of CD63 and CD203c but recently, CD63 expression has been shown to result from only the anaphylactic degranulation form of histamine release (MacGlashan, 2012).

Performance comparison between CD63 and CD203c in confirmed IgE-mediated amoxicillin allergy, the sensitivity for CD203c was found to be far superior to that of CD63 (60 versus 20 %) (Abuaf *et al*., 2008). However, opposite finding from the same group also demonstrated that CD63 expression was upregulated more frequently than CD203c in patients with non-allergy NSAID hypersensitivity (Abuaf *et al*., 2012).

Generally, the claimed superior performance of CD203c has been questioned in more than one study with comments that the presently widely used basophil activation monitored by expression of CD63 is a validated test while the more recently introduced marker requires more extensive study and validation for different clinical conditions (Knol *et al*., 1991). Some have claimed that CD203c produces slightly improved sensitivity if not by itself then together with CD63 (Sturm *et al*., 2010). The use of both markers has been advocated, and the practice of using dual markers now seems to be common (Christensen *et al*., 2013; Mikkelsen *et al*., 2010). More recently identified basophil activation markers like CD13, CD107a, and CD164 maybe the forerunners of a second generation of BATs (Hennersdorf *et al*., 2005).

CHAPTER 3: MATERIALS AND METHODS

3.1 Study design

This is a case control study comprising patients who were diagnosed as allergic to beta-lactam antibiotics by doctors/clinicians based on European Network for Drug Allergy (ENDA) 2003 Guidelines: Immediate allergic reactions to beta-lactam antibiotic and controls that had no history of any drug allergy.

3.2 Study populations

Fifty subjects comprising 25 patients diagnosed as immediate allergic reactions to beta-lactam antibiotics as defined by the European Network for Drug Allergy (ENDA) 2003 Guidelines; whom referred to the Allergy Clinic, Hospital Kuala Lumpur (HKL) and 25 healthy controls defined by individuals with no history of drug allergy including the beta-lactam antibiotics and negative to Skin Prick Test (SPT) were assessed. A comprehensive clinical evaluation for diagnosing beta-lactam allergy were performed using ENDA recommended clinical questionnaire (Appendix A). The current study was approved by Medical Research of Ethics Committee of the Ministry of Health in Malaysia (KKM/NIHSEC/P13-901, NMRR-13-922-17589), informed consent for all testing were obtained from all patients and controls (patient information sheet and informed consent form are attached in Appendix B (English version) and Appendix C (*Bahasa* version)).

3.2.1 Selection criteria for patients and controls

The patients and controls were enrolled according to predetermined inclusion criteria. The inclusion criterion in the study was diagnosed as allergy to beta-lactams by doctors (or clinicians) and consented to participate voluntarily in the study. Patients who received anti-IgE and anti-histamines within 24 hours of consultation and with other

drug allergic reaction were excluded. Patients clinical manifestation were assessed based on clinical patterns described by ENDA Guideline 2003.

Table 3.1: Clinical criteria for diagnosis of anaphylaxis (Sampson *et al*., 2006)

Anaphylaxis is highly likely when any one of the following three criteria are fulfilled:
1. Acute onset of an illness (minutes to several hours) with involvement of the skin, mucosal tissue, or both (e.g. generalized hives, pruritus or flushing, swollen lips-tongue-uvula) and at least one of the following: a. Respiratory compromise (e.g. dyspnea, wheeze-bronchospasm, stridor, reduced Peak Expiratory Flow (PEF), hypoxemia) b. Reduced blood pressure (BP) or associated symptoms of end-organ dysfunction (e.g. hypotonia [collapse], syncope, incontinence)
2. Two or more of the following that occur rapidly after exposure to a likely allergen for that patient (minutes to several hours): a. Involvement of the skin-mucosal tissue (e.g. generalized hives, itch-flush, swollen lips-tongue-uvula) b. Respiratory compromise (e.g. dyspnea, wheeze-bronchospasm, stridor, reduced PEF, hypoxemia) c. Reduced BP or associated symptoms (e.g. hypotonia [collapse], syncope, incontinence) d. Persistent gastrointestinal symptoms (e.g. crampy abdominal pain, vomiting)
3. Reduced BP after exposure to known allergen for that patient (minutes to several hours): a. Infants and children: low systolic BP (age specific) or greater than 30% decrease in systolic BP* b. Adults: systolic BP of less than 90 mmHg or greater than 30% decrease from that person"s baseline

* Low systolic blood pressure for children is defined as less than 70 mmHg from 1 month to 1 year, less than (70 mmHg + [2 X age]) from 1 to 10 years, and less than 90 mmHg from 11 to 17 years.

Based on the guidelines, the two main entities of drug allergy clinical manifestations generally recognized are urticaria, with or without angioedema and anaphylaxis. The first stage of anaphylaxis is manifested by urticaria developed at different sites of the body. According to Sampson *et al*. (2006), anaphylaxis is defined as a rapid onset of severe allergic reaction that often lead to death. Anaphylaxis can be diagnosed by the clinical criteria proposed in Table 3.1. Bronchial asthma and/or rhinitis are primarily manifested during allergic reactions to beta-lactam antibiotics elucidating the special sensitivity of various affected organ.

3.3 Skin Prick Test (SPT)

The SPT was performed according to the conventional techniques (Berger, 2002) using ALK Abelló (Madrid, Spain) lancets. The list of freshly prepared beta-lactam drug allergens used for the testing with respective concentrations were shown in Table 3.2. Histamine hydrochloride (10 mg/ml) was used as positive control and 90% saline solution as negative control. All allergens and controls were purchased from Diater LABORATORIOS S.A. The SPT is considered positive when the wheals diameter equals to 3 mm or greater than the negative control within 20 minutes. The technique used for SPT involves administration of a drop of diluted drug allergen and skin puncturing using calibrated lancet (1 mm) held vertically at an angle of 45°. Within ten minutes itchy wheal should develop at positive control site. The maximum or mean diameter of the wheals to various allergens should be read at 15 minutes. Positive response is indicated by a wheal diameter of 3 mm or more which indicate allergen sensitization. In order to exclude dermographism, negative control is important to facilitate test interpretation. SPT interpretation should be done in the context of patients‟ clinical history. Positive results can occur in people without symptoms and, similarly, false negative results may occur (Berger, 2002).

Table 3.2: Beta-lactam allergens used for SPT

Beta-lactam allergens	Concentration
Benzylpenicilloyl octa-L-lysine	0.04 mg/ml
Sodium benzylpenilloate	0.50 mg/ml
Sodium amoxicillin	20.00 mg/ml
Potassium clavulanate	20.00 mg/ml

3.4 Laboratory testing

A total of 7 ml of peripheral blood in ethylene-diamine tetra acetic acid (EDTA) tubes was collected from all participants during an arranged appointment with the clinicians. Whole blood samples were tested for CD63 expression using basophil activation test (BAT) and specific IgE antibodies were measured by the CAP®-Fluorescence Enzyme Immunoassay (CAP®-FEIA).

3.4.1 Specific IgE quantification

Quantification of specific IgE antibodies against Penicillin G, Penicillin V, Ampicillin and Amoxicillin were performed by FEIA method using UniCAP® Phadia 250 systems (Thermo Fischer Scientific, Uppsala, Sweden), using c1 (Penicilloyl G), c2 (Penicilloyl V), c5 (Ampicilloyl) and c6 (Amoxilloyl) following manufacturer‟s instructions. Briefly, the beta-lactams were covalently coupled to ImmunoCAP® to react with the specific IgE in the plasma sample. After washing away the non-specific IgE, enzyme-labelled antibodies against IgE were added to form a complex. In a second step after incubation and washing, an incubation with a developing agent was carried out, after stopping the reaction, the fluorescence of the elute was measured. The results were

obtained by direct comparison with standards run in parallel, with the value of specific IgE ≥ 0.35 kUA/L were considered as positive.

3.4.2 Basophil Activation Test (BAT)

The flow cytometric analysis of the *in vitro* activated basophils with Flow CAST® technique (Bühlmann Laboratories TM, Switzerland) was performed. The assay is based on the method firstly described by (Sainte-Laudy *et al*. 1994 and 1996) where basophil activation by allergens or controls is detected by flow-cytometry measured by the increase of the CD63 (gp53) at the cellular surface. For each test, eight test tubes were used, each containing 50 µl of whole blood collected on EDTA. The cell stimulation was performed immediately after collection of blood and plasma for specific IgE immunoassays. The first sample was mixed with 50 µl stimulation buffer as negative control, and next sample was the positive controls mixed with 25 µl solution of anti-FcεRI (a highly specific monoclonal antibody for the IgE receptor) and 25 µl solution of fMLP (a non-specific cell activator-the chemotactic peptide N-Formyl-Met-Leu). The other six samples were mixed with commercially available antibiotics allergen (CAST® Bühlmann Allergens) namely Benzylpenicillyl-polylysine, (PPL), Minor determinant mix (MDM), Penicillin G, Penicillin V, Amoxicillin and Ampicillin with respective concentration shown in Table 3.3.

Anti-CCR3-PE (human chemokine receptor labeled with phycoerythrin) and anti-CD63-FITC (a glycoprotein expressed on activated basophils) were used as staining reagents were added in each of the test tube. After an incubation period of 15 minutes at 37˚C in a water bath, 2 ml of pre-warmed lysing solution was added to each tube and incubated for 10 minutes at room temperature. After centrifuging and washing, the cells were suspended in 500 µl wash buffer.

Table 3.3: Beta-lactam allergens used for basophil stimulation and concentrations

Beta-lactam allergens	Concentration
Benzylpenicilloyl-Polylysine (PPL)	50.0 µg/ml
Minor Determinant Mix (MDM)	1.0 mg/ml
Penicillin G	4.0 mg/ml
Penicillin V	4.0 mg/ml
Ampicillin	10.0 mg/ml
Amoxicillin	2.5 mg/ml

The quantification of the increase CD63 marker on basophils was detected using CellQuest software (FACSCalibur BD Analyser). The number of event acquired was set to contain at least 400 basophils (expressing CCR3- PE). The gate was set by including the entire basophil population CCR3 positive cells with low Side Scatter (SSC low) and the calculated the percentage of CD63 positive cells was compared to the total amount of basophils gated. The result was considered positive when the percentage of the activated basophils was 5% or more over the spontaneous activation observed for the negative control. The stimulation index calculated as the ratio between the percentage of activated basophils with the allergens and negative control was $\geqslant 2$.

3.5 Statistical analysis

The statistical analysis was performed in Excel software (2016, Microsoft Corporation TM, Seattle USA). Cohen‟s Kappa Index (k) was used to assess the agreement between BAT and FEIA. Cohen‟s Kappa Index was calculated as k=(pa-

pe)/(1-pe), where: pa= proportions of observation in agreement; pe=proportions of agreement due to chance.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Patient's clinical history

A total of 25 patients recruited within 24 months of reactions occur to the time of recruitment. Patient‟s clinical manifestations with Skin Prick Test (SPT), immunoassay and Basophil Activation Test (BAT) results are shown in Table 4.1. Of 25 referred patients, 14 were females and 11 were males, with age range between 16 to 75 years. Clinical manifestations include angioedema in 14 cases (56%), anaphylactic symptoms in 7 cases (28%), urticaria in 4 cases (16%), diarrhea in 2 cases (8%), followed by vomiting, macular exanthema, pruritis, bronchospasm, shortness of breath and hypotension with one case (4%) for each.

4.2 Skin Prick Test (SPT)

Of 25 patients, only one patient (patient 23) was skin-test positive to Amoxicillin and to the in-house preparation Ampicillin drug (not shown in Table 4.1). However, SPT to one patient was not perform due to consent matter. Diagnosis of beta-lactam allergy is always based on SPT and intradermal tests. Sensitivity of skin tests, however, does not exceed 50 to 70%. The sensitivity of SPT in the diagnosis of immediate reaction to beta-lactams mainly depends on the drug that elicited the reaction and the time elapsed from the clinical reaction. In the current study, one patient (patient 23) was determined as skin test-positive to Amoxicillin and to in-house Ampicillin preparation but showed good tolerance to Penicillin G and Penicillin V, these results were also supported by BAT results (Figure 4.4). This suggest that this patient may be particularly sensitized to aminopenicillin with good tolerance to parent drug. The side chains (R substituents) of natural penicillins (Penicillin G and Penicillin V) and aminopenicillins (Amoxicillin and Ampicillin) are shown in Figure 4.1.

Table 4.1: Patient"s clinical manifestations with SPT, immunoassay and BAT results

				SPT			ImmunoCAP® FEIA (kUA/L)				BAT (SI)					
Patient	**Age**	**Sex**	**Clinical manifestations**	**PPL-MDM**	**Amox**	**Clav A**	**Pen G**	**Pen V**	**Amp**	**Amox**	**Pen G**	**Pen V**	**PPL**	**MDM**	**Amp**	**Amox**
1	75	F	Diarrhea & vomiting	-	-	-	0	0	0.03	0.03	1	1	1	1	1	1
2	36	M	Pruritis	-	-	-	0	0.02	0.04	0.07	1	1	1	1	1	1
3	30	F	Urticaria & macular exanthema	-	-	-	0.36	0.31	0.39	0.56	1	1	1	1	1	1
4	25	F	Diarrhea	-	-	-	0	0	0.04	0.04	1	1	1	1	1	1
5	23	F	Angioedema	-	-	-	0.02	0.05	0.06	0.15	1	1	1	1	1	1
6	25	F	Urticaria	-	-	-	0.01	0.03	0.04	0.11	1	1	1	1	1	1
7	28	F	Angioedema	-	-	-	0	0.04	0.03	0.06	1	1	1	1	1	1
8	30	M	Angioedema & mild anaphylaxis	-	-	-	0.01	0	0.03	0.05	1	1	1	1	1	1
9	43	F	Anaphylactic shock	ND	ND	ND	10.7	10.8	8.29	6.77	40	100	1	1	79	1
10	33	F	Anaphylaxis perioperative	-	-	-	0	0	0.04	0.05	1	1	1	1	1	1
11	40	F	Angioedema	-	-	-	0	0.01	0.08	0.10	1	1	1	1	1	1
12	65	M	Angioedema	-	-	-	0.03	0.04	0.10	0.11	1	1	1	1	1	1
13	60	M	Anaphylaxis	-	-	-	0.49	0.31	0.52	0.74	1	1	1	1	1	1

Table 4.1, continued

Patient	Age	Sex	Clinical manifestations	SPT			ImmunoCAP® FEIA (kUA/L)				BAT (SI)					
				PPL-MDM	Amox	Clav A	Pen G	Pen V	Amp	Amox	Pen G	Pen V	PPL	MDM	Amp	Amox
14	52	F	Angioedema	-	-	-	0	0.06	0.08	0.13	1	1	1	1	1	1
15	58	F	Angioedema	-	-	-	0	0.04	0.08	0.11	1	1	1	1	1	1
16	16	M	Anaphylaxis	-	-	-	0.05	0.06	0.17	0.15	1	1	1	1	1	1
17	26	M	Anaphylaxis, angioedema, urticaria & bronchospasm	-	-	-	0.03	0.04	0.08	0.08	1	1	1	1	1	1
18	35	M	Shortness of breath	-	-	-	0.01	0.02	0.08	0.06	1	1	1	1	1	1
19	43	F	Urticaria & angioedema	-	-	-	0	0	0.03	0.08	1	1	1	1	1	1
20	37	M	Angioedema	-	-	-	0.11	0.02	0.09	0.03	1	1	1	1	1	1
21	39	M	Hypotension	-	-	-	0.17	0.25	0.36	0.56	1	1	1	1	1	1
22	59	F	Angioedema	-	-	-	0.06	0.13	0.13	0.27	1	1	1	1	1	1
23	38	M	Anaphylaxis & angioedema	-	+	-	0	0	0.03	0.02	1	1	1	1	82	59
24	32	M	Angioedema	-	-	-	0.01	0.10	0	0.05	1	1	1	1	1	1
25	22	F	Angioedema	-	-	-	0.04	0.01	0.06	0.08	1	1	1	1	1	1

SPT: Skin Prick Test; BAT: Basophil Activation Test; ND: not done; Pen G: Penicillin G; Pen V: Penicillin V; Amp: Ampicillin; Amox: Amoxicillin; Clav A: Clavulanic acid; PPL: benzylpenicilloyl-polylysine; MDM: minor determinant mix; „+“: positive result; „- „: negative result

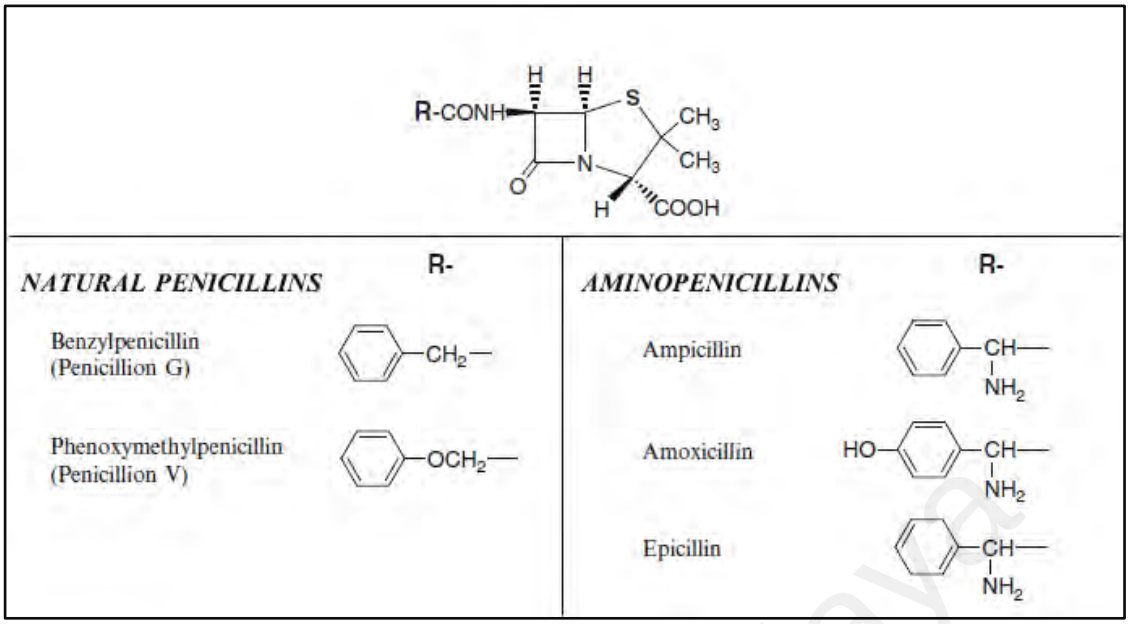

Figure 4.1: Structure of different side chain (R) groups on individual penicillin grouped according to structure similarities (Source: Baldo & Pham, (2013)).

Torres *et al*. (2003) reported that the side chain seems to play a role as antigenic determinant in some allergic reactions to beta-lactams. In the report, skin testing of 20 patients with history of beta-lactam allergy using Penicilloyl-Polylysine (PPL), Minor Determinant Mix (MDM), Amoxicillin and Ampicillin showed all patients were allergic to only Amoxicillin and Ampicillin.

The immunoglobulin E (IgE) antibodies in the sera of patients allergic to beta-lactam antibiotics may detect a range of specificities not only to the entire penicillin molecule but also to the side chain. The structural heterogeneity of allergic determinants cause IgE antibodies in the patient sera show heterogenous recognition response.

Immunochemical study by Zhao *et al.* (2001) performed in sera of patients allergic to penicillin demonstrated selective and unexpected reactions to Amoxicillin. IgE antibodies reactive to amoxicilloyl determinants were demonstrated in one subject while IgE from another subject demonstrated multiple reactivity to penicilloyl and penicillanyl determinants of different penicillins but not with the amoxicilloyl determinant. Their experiment on hapten inhibition had revealed that the combining

sites of amoxicilloyl reactive IgE antibodies were complementary to Amoxicillin in such a way that allows binding to the hydroxyaminobenzyl side-chain and the thiazolidine ring carboxyl. Thus, these fit the conditions of drug in '-oyl' form which involves linkage through the 2-carboxyl of the thiazolidine ring. When adsorption study was performed on the second serum, wide range of IgE reactivity occurred due to single IgE antibodies population detecting common specificity on the different penicillins. Recognition to the benzyl portion of the side-chain of benzylpenicilloyl, benzylpenicillanyl, ampicilloyl, ampicillanyl and amoxicillanyl determinants were also demonstrated when there is free antibody access but not to the hydroxyl ring in Amoxicillin. Inhibited access may occur when there is opening of the beta-lactam ring allows increased flexibility and rotation of the molecule that lead to the conjugation of Amoxicillin in „-oyl‟ form. This form is closely associated to the hydroxyaminobenzyl side-chain of Amoxicillin with the linked peptide carrier. In such close steric association, hydrogen-bonding involving the ring hydroxyl and amino acids of the carrier may prevent antibody access to the side-chain region of the amoxicilloyl determinant.

A study by Harle and Baldo (1990) employing quantitative immunochemical direct binding and inhibition immunoassay using penicillin-solid phase complexes demonstrated that IgE recognized different penicillin side chain substituents. Preferential recognition to Ticarcillin was observed although IgE antibodies also bound to other regions of penicillin structures. That could be due to a population of penicillin-reactive IgE antibodies recognizing the R substituent in Ticarcillin. The recognition of side chain as allergic determinants by some patients indicate the importance to include different individual penicillin in selecting reagents for skin testing.

Elseviers *et al*. (2007) in their study reported a decrease of skin test sensitivity using benzylpenicillin determinants. The percentage of positive skin test results to PPL

and/or MDM has decreased from 77.7 to 42.1%. Such findings could be due to change in sensitization patterns secondary to an increase in the usage of other beta-lactams such as aminopenicillins, associated with a concomitant decrease in the consumption of benzylpenicillin in many countries. These results again suggest the importance of using penicillin determinant and other beta-lactams determinants to correctly diagnose immediate reactions to beta-lactam because of changes in prescribing habits.

4.3 Basophil CD63 expression and usefulness in detecting IgE-mediated beta-lactam allergy

In the current study, all 25 patients were evaluated for BAT. Example of optimal basophil gating is shown in Figure 4.2. Two patients (patients 9 and 23) demonstrated highly expressed CD63-FITC (Fluorescein isothiocyanate) shown in Figure 4.3 and Figure 4.4, respectively. Patient 9 positively expressed CD63 on its activated basophil when tested with Penicillin G (Pen G) (10.18%, SI=40), Penicillin V (Pen V) (25.07%, SI=100) and Ampicillin (19.52%, SI=79). The results are consistent to its immunoassay results except for Amoxicillin. Patient 23 positively expressed CD63 on activated basophil tested with Ampicillin (82.32%, SI=82) and Amoxicillin (59.27%, SI=59). Interestingly, patient 23 was tested negative in immunoassay (sIgE $\leq$ 0.35 kUA/L) to the entire drug tested. Out of 25 patients, only two were BAT positive resulting in a sensitivity of 8% (2/25) and all 25 controls were tested negative resulting in a specificity of 100%. Fair agreement between BAT and sIgE results was observed (Cohen Kappa Index = 0.25).

The present study also revealed two patients positively expressed CD63 on activated basophil. Both patients experienced anaphylactic symptoms, however, only one patient (patient 9) shows consistent results in BAT and immunoassay as opposed to another patient (patient 23). As suggested earlier, patient 23 may have particularly

sensitized to side chain of Amoxicillin and Ampicillin as shown in BAT and SPT results, but this was not observed in his/her immunoassay results.

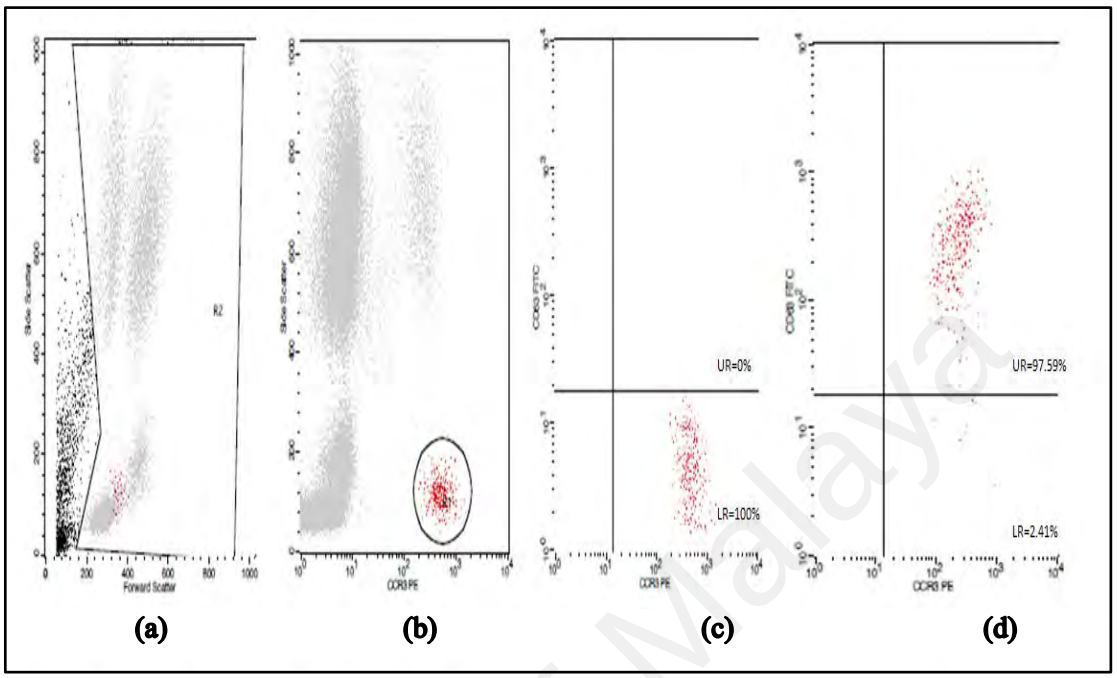

Figure 4.2: Example of optimal basophil gating with (a) gated area shows white blood cells; (b) circle area shows CCR3-PE positive cells; (c) negative control (stimulation buffer); (d) positive control (anti FcεRI + fMLP).

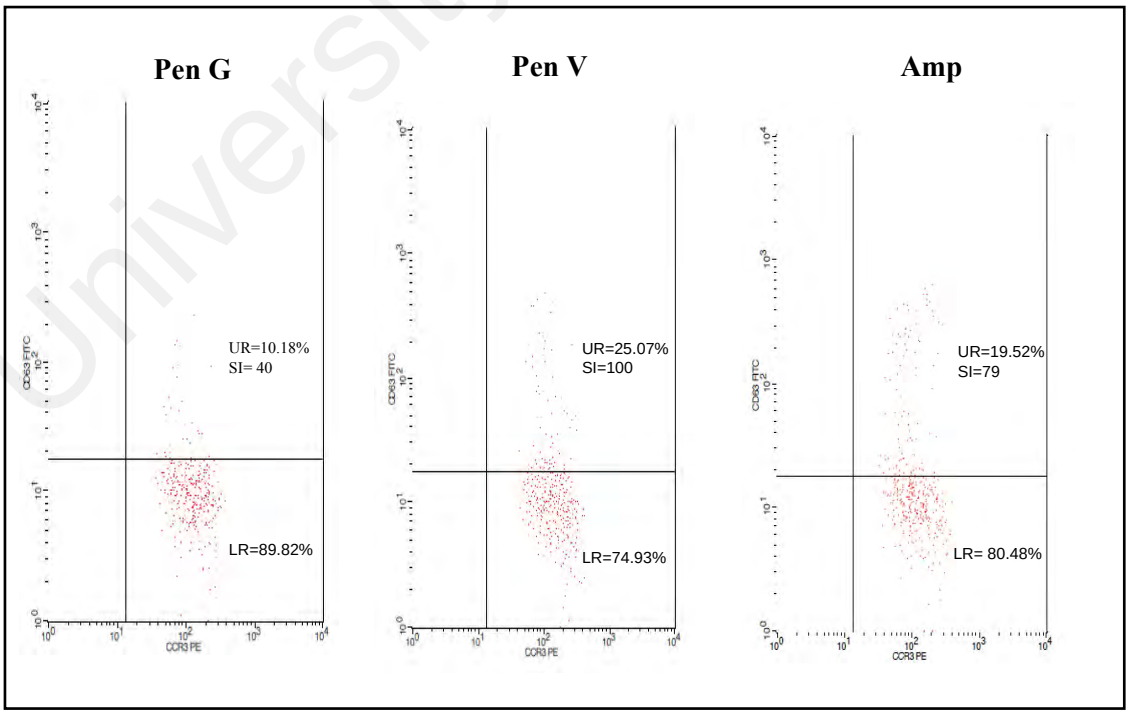

Figure 4.3: CD63-FITC expression on activated basophil in patient 9 tested with Pen G (10.18%, SI=40), Pen V (25.07%, SI=100) and Amp (19.52%, SI=79).

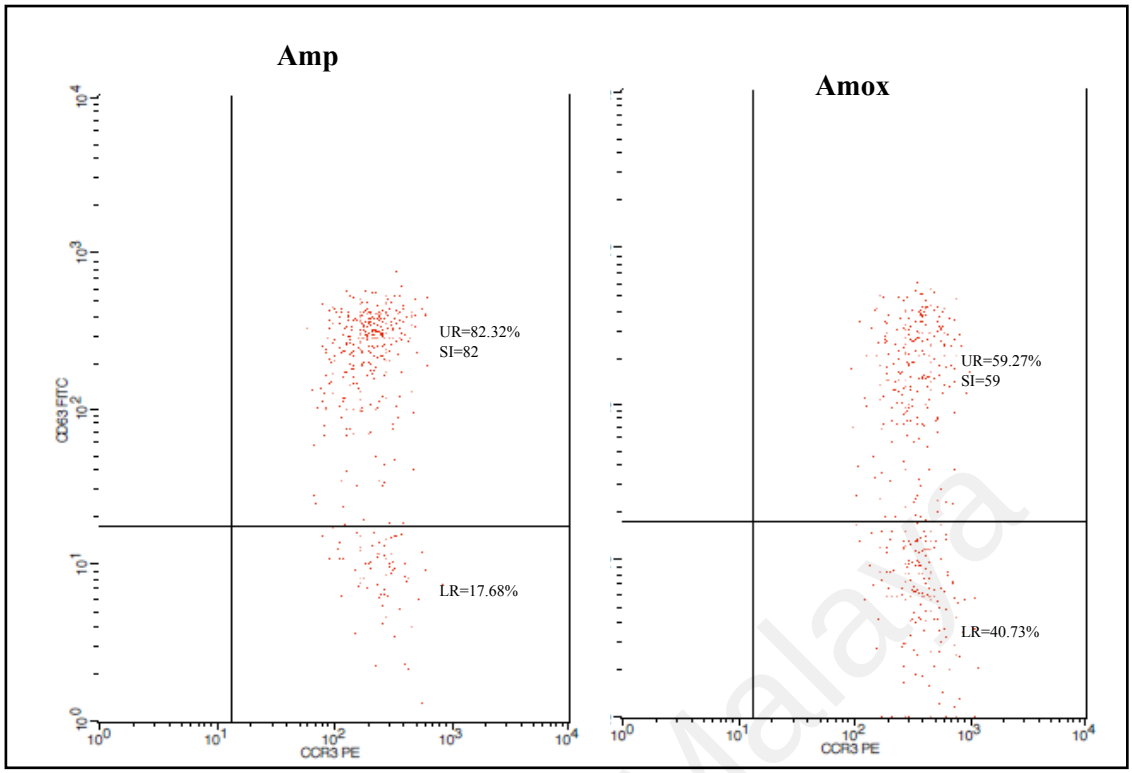

Figure 4.4: CD63-FITC expression on activated basophil in patients 23 tested with Amp (82.32%, SI=82 and Amox (59.27%, SI=59).

On the other hand, BAT results of three patients (patients 3, 13, and 21) were negative despite positive immunoassay results. Nevertheless, although positive, sIgE level of these patients were between low and moderate, low (sIgE level: 0.35-0.69 kU_A/L) and moderate (sIgE level: 0.70-3.49 kU_A/L) compare to patient 9 with high sIgE level (sIgE level: 3.5-17.49 kU_A/L). Such incidence remarks the importance of sIgE level in basophil activation. Theoretically, degranulation of basophils is definitive event in allergy, whereas antigen-specific IgE only serves as one of the key messengers. Hence, patients with clinical manifestations of allergies exhibiting elevated levels of sIgE but does not show positive basophil degranulation may be experiencing IgE-independent allergic reactions (He *et al*., 2013).

In cases where symptomatic patients show negative results in both immunoassay and BAT (n=21), these patients may have undergone sIgE immunoassay and/or BAT negativization. It was shown that level of sIgE tend to decrease over time in patients with immediate allergic reactions to amoxicillin (Fernandez *et al*., 2009). In addition, negativization rate also differs between patients with cross-reactivity and those with selective IgE response (Blanca *et al*., 1999). Because of the loss of sensitivity over time, the determination of sIgE to penicillin in patients with immediate allergic reactions must be done as soon as possible after the reaction. Sensitivity of BAT for evaluating immediate allergic reactions to drugs may also decrease over time (Sanz *et al*., 2009). Nevertheless, sensitivity of the test can be improved if patients are tested soon upon allergic reaction (Abuaf *et al*., 2008). It was shown that usefulness of BAT is markedly increase when allergic reactions are investigated within 2 years (Hagău *et al*., 2010).

As reported by Kleine-Tebbe *et al*. (2006), several variables determine individual basophil outcomes, includes (a) the total density IgE receptor on cell surface; (b) the proportion of membrane-bound allergen-specific IgE antibodies versus total IgE; (c) the intrinsic cellular sensitivity of basophils, i.e. determined by the number of IgE molecules required for 50% of maximal cellular responses; (d) the cellular reactivity defined as the maximal cellular response upon optimal stimulation; (e) the allergen structural features determining the number and respective distances of epitopes able to bind to IgE on a single allergen molecule and in a mixture of allergenic molecules; (f) the nature of the complexes formed by allergens and IgE (dimers, trimers, oligomers); (g) the duration of contact between allergen and membrane-bound IgE; and (h) the presence of specific Immunoglobulin G (IgG) competing with IgE for allergen binding. Some of these parameters are interdependent or connected with serological parameters, while others are not. Although these rules for immediate allergic reaction may not be essentially valid for drug allergy it may have affect basophil activation in these patients.

High-affinity IgE receptor on basophil surface, FcεRI (Fc region of immunoglobulin E I)-mediated signaling requires aggregation of cell-surface antigen-specific IgE bound to this receptor (Turner & Kinet, 1999). The antigen-IgE reaction can be pictured as the two-dimensional reaction where the aggregation requirements can be subtle because the actual juxtaposition of two or more receptors is not strictly required. Although higher aggregate sizes appear more efficacious, simple dimers of FcεRI can induce signaling. In the simplest case of bivalent symmetric antigen, the shape of the dose response in an aggregation reaction is very similar to a bell-shaped curve. However, the complexity of antigens and the relative affinity of different epitopes for different profiles of epitope-specific IgE can results a variation of dose response curves. Tests with single concentrations of antigen can be ambiguous. The affinity of antigen for the IgE determines the point of optimum activation, so that this point might vary significantly among subjects. Thus, best studies include as broad a dose response as feasible given the constraints typical for venipuncture and the time to do an experiment (MacGlashan, 2013).

The sensitivity of BAT in the present study can be regarded as too low (8%) but in range with other studies (0%) involving immediate type fluoroquinolone hypersensitivity (Lobera *et al*., 2010; Seitz *et al*., 2009). BAT performance in detecting beta-lactam antibiotic allergy varied between groups with sensitivities ranged from 28.6 to 55%. Other studies using CD63 as an activation marker in a different protocol, although still low, demonstrated higher results (sensitivity up to 55%) (Eberlein *et al*., 2010; Torres *et al*., 2010). Several large-scale studies have consistently demonstrated the sensitivity to be approximately 50%, in patients with positive clinical history and skin tests (De Week *et al*., 2009; Gamboa *et al*., 2004; Sanz *et al*., 2002). Sensitivity of BAT was reported to be approximately 10% higher than immunoassay and specificity more than 90%, but not to skin testing. Thus, due to high specificity of the test, positive

BAT results were clinically significant (Song & Chang, 2013). Skin testing and BAT are not always confirming each other, which also shown in present study Only 50 to 60% of skin test-positive patients shows BAT positive and up to one third of skin test-negative patients were identified by BAT (De Week *et al*., 2009; Torres *et al*., 2010). Current study showed only one (4%) skin test-positive patient (patient 23) had positive BAT.

The variance in BAT sensitivity are possibly contributed by patient‟s selection criteria for example the severity of the reactions and time length since the reaction (optimum: one to six months) and performance of the test. Technical variations such as different activation time (range 20 to 40 minutes) and different activation markers (CD63 and/or CD203c) has additionally complicate the comparison among results. BAT is usually performed from either heparinized, citrate or EDTA-anticoagulated whole blood. When EDTA is used, anticoagulant Ca^{2+} has to be supplemented to enable proper degranulation but this was not done in our experiment (Steiner *et al*., 2016).

Importantly, in this study, BAT was positive in both patients where SPT cannot be performed or when sIgE are negatives. These results suggested that BAT should be performed in cases where the diagnosis of drug allergy is highly suspected especially in anaphylaxis case but it is not supported by results of SPT or *in vitro* IgE measurements. In addition, by performing BAT, potential life-threatening provocations tests can be avoided. This study of patients with type-I beta-lactam allergy performed within 2 years after allergic reaction shows fair agreement between sIgE level and BAT results (Cohen Kappa Index = 0.25). The agreement is parallel to other study relative to history plus skin test and BAT (0.35) where the investigators then observe lower agreement (0.25) when BAT is performed after 2 years (Hagău *et al*., 2010). Thus, the concordance between sIgE and BAT may be higher when the time interval is short. However, the clinical utility of BAT, remains restricted by the requirement for fresh blood, specific

laboratory equipment and technician time and thus this test remains largely as research tool until its role can be fully defined (Mirakian *et al*., 2015).

4.4 sIgE immunoassay for beta-lactam allergy

In FEIA, of the 25 patients, four were sIgE positive resulting in a sensitivity of 16% (4/25) and all 25 controls were tested negative resulting in a specificity of 100%. Three (patients 3, 13 and 21) of four patients with positive immunoassay to at least one of the drug tested has negative BAT results. The sIgE levels of the three patients were between low (sIgE level: 0.35-0.69 kU_A/L) and moderate (sIgE level: 0.70-3.49 kU_A/L) and therefore may not be detected by considerably low sensitivity of BAT. Another patient (patient 9) with high (sIgE level: 3.50-17.5 kU_A/L) level of sIgE antibodies showed positive BAT with consistency in all the drugs tested in immunoassay except for Amoxicillin.

The present study also revealed higher immunoassay sensitivity (16%) compare to BAT (8%). One (patient 9) of the four patients with positive immunoassay to at least one of the drug tested, had positive BAT results. Interestingly, of all sIgE positive patient no one has positive skin test except for one patient that SPT was not performed. Patient 23 on the other hand, had negative immunoassay albeit positive results in BAT and SPT indicating low sensitivity of the assay. Several evidence of low sensitivity and specificity of the immunoassay may explain the negative sIgE result despite positive BAT and SPT (Košnik *et al*., 2013). Depending on clinical manifestations, immunoassay sensitivity was between 0 to 25% (Fontaine *et al*., 2007). In a previous study by Hjortlund *et al*. (2013), showed that, of 19 sIgE positive patients, only six were positive in intradermal skin testing. Moreover, they observed no correlation between sIgE positive patient and the culprit drug reported by patients.

In a study by Macy *et al.* (2010) to determine if sIgE testing can replace skin test or oral challenge, conclude that the assays are not useful in diagnosing penicillin allergy in patients and skin testing and/or oral challenge remain the criterion standard test to determine the tolerance of the drug.

In type I immediate hypersensitivity reactions, the IgE antibodies recognize mainly the original penicillin structure with a variable degree of cross-reactivity with other structures (Solensky *et al*., 2002). Additionally, negativization of sIgE occur significantly earlier when tested using BAT and this was shown in decreasing of sIgE immunoassay sensitivity over time especially in immediate allergic to Amoxicillin (Fernandez *et al*., 2009). In the current study because patient 23 had positive BAT but negative immunoassay, IgE negativization may not have occur, but different hapten may cause activation of basophil. On the other hand, negativization may also have occur in the three patients (patients 3, 13, and 21) with moderate levels of sIgE but negative to BAT.

Study by Vultaggio *et al*. (2015), demonstrated that the ratio of the sum of beta-lactam specific to total IgE is able to improve the diagnostic performance of immunoassay in identifying allergic patients. An increase specific/total IgE ratio may be correlated with a high probability that beta-lactam specific IgE on basophils or mast cells are very close. This case is rare when the ratio is low. Their data also suggest the ratio are clinically useful in patients with serum total IgE > 200 kUA/L. Unfortunately, we did not perform total IgE quantification in the current study.

Quantification of beta-lactam sIgE antibodies is an important complementary information. However, clinically validated tests for drug sIgE, are difficult to develop, require complex coupling reactions for attaching the drug hapten onto solid phase for antibody recognition, are only available for limited number of antibiotics (Steiner *et al*., 2016).

In a non-specialist setting, sIgE immunoassay is the only non-acute test that can be undertaken. For alternative tests, such as SPT or drug challenges the individual would need to be referred to specialist drug allergy services for investigation. Based on the inspection of the paired sensitivity and specificity forest plots of several studies by the National Institute for Health and Care Excellence (United Kingdom), there is a serious inconsistency in the measurement of serum sIgE for beta-lactam antibiotic observed (Blanca *et al*., 2001; Fontaine *et al*., 2007; Qiao *et al*., 2005; Silva *et al*., 2009; Vultaggio *et al*., 2009). High variables results were observed in terms of sensitivity and specificity from the observational studies (n=1624). Generally, the specificity is higher than sensitivity indicating that immunoassay is better in „ruling in" than „ruling out" beta-lactam allergy. However, due to high imprecision drawing a clear conclusion of the test accuracy is rather difficult (National Institute for Health and Care Excellence (UK), 2014).

Thus, considering the inconsistent sensitivity of laboratory IgE testing, IgE testing should be considered only in selected patients who undergoing specialist investigation together with skin tests. IgE tests can be useful in cases with severe anaphylaxis to limit drug provocation, particularly if skin tests are unexpectedly negative.

4.5 Limitation of study

This study involved considerably low number of patients (n=25) involving only two centers (Allergy Clinic, Hospital Kuala Lumpur and Dermatology Department, Hospital Kuala Lumpur). Sufficient number of cases and controls are necessary to provide more useful information on the utility of the test in diagnosing allergies to beta-lactam. Also, in our center (Allergy Clinic, Hospital Kuala Lumpur), SPT was performed using limited commercially available drug, which in-house drug were added in the other center (Dermatology Department, Hospital Kuala Lumpur) allowing wider

chance of detection. BAT and FEIA were carried out using limited commercially available beta-lactam antibiotics (BAT; PPL, MDM, Pen G, Pen V, Amp, & Amox: FEIA; Pen G, Pen V, Amp & Amox) available from respective manufacturer. Drug allergens are more varied than food and inhalant allergens, therefore complex preparation of drugs to mimic the metabolites occur *in vivo* are suggested in future study. Expansion of validated beta-lactam drug allergens available for BAT and FEIA may allow detail comparison of both tests.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

The current study aimed to determine the level of CD63 expression in the activated basophil cells in patients with beta-lactam allergy by flow-cytometry method and secondly, to compare the serum level of specific IgE (sIgE) antibodies and CD63 expression in patients with beta-lactam allergy. This study was conducted with hopes to figure out the best diagnostic test in term of sensitivity and specificity to correctly diagnose the patients and thus prevent unnecessary avoidance drugs and ultimately reduce the number of patient that have to undergo drug provocation tests. The study showed that diagnosis of patients allergic to beta-lactam based on clinical history and Skin Prick Test (SPT) using limited commercially available drug were inadequate. In regard to sensitivity, immunoassay by Fluorescence Enzyme Immunoassay (FEIA) was shown to be more sensitive compared to Basophil Activation Test (BAT) despite of high specificity of both tests. CD63 were highly expressed in patient with high level of sIgE antibodies against the culprit drugs, but not in patient with moderate level of sIgE. Patient who did not positively expressed CD63 but has moderate level of sIgE antibodies (patients 3, 13 and 21) showed similar clinical manifestations. Nevertheless, BAT is still useful in preventing misdiagnosis and unnecessary avoidance of the drugs especially in patients where both SPT and immunoassay were negatives or cannot be performed.

BAT using CD63 as an activation marker is more likely to be a promising diagnostic tool for clinical decisions regarding patients with drug allergy. The main advantage of BAT over conventional diagnostic tools is the capability to assess multiple drug simultaneously, safely and specifically. Since it allows simultaneous testing of different drugs, BAT can contribute in the identification of cross-reactive

substances/safe therapeutic alternatives. BAT is not a primary diagnostic tool, it is a complementary to the skin test and quantification of allergen-sIgE. Applications of CD63 as expression marker are rapidly extending into diagnosing allergies caused by various other drugs. Currently, it is mostly applied in the diagnosis of allergy to beta-lactam antibiotics and particularly useful to confirm clinical suspicion without performing dangerous provocation tests in cases where no alternative test is available. However, in order to obtain optimal predictive values, it is mandatory to apply drug-specific decision thresholds that can be obtained from receiver operating characteristic-analysis between well-defined patients and exposed controls.

Another advantage of this technique comes from its obvious features that form part of the *in vivo* response to allergenic challenge resulting from the release of mediators. Reliable results can be obtained by direct examination of basophil normal in the presence of other cells (Baldo & Pham, 2013). However, at present, the procedures shortcomings, particularly its sensitivity and diagnostic accuracy, need to be kept in mind. A better understanding of relationships between the expression of extra and intracellular activation markers and the release of mediators may significantly improve diagnostic performance. Application of recent methodology may also lead to an understanding of the underlying intracellular signaling mechanisms of drug and other agent-induced degranulation of basophils. BAT will continue to be widely applied in both pure and crude form of allergen examination as well as allergoids, vaccines, newly introduced drugs, additives, and recombinant preparations, where other tests are not available or not suitable. Accumulation of knowledge on basophil markers and other routes of activation, BAT might be an appropriate and valuable procedure for the study and diagnosis of some IgE-independent drug reactions.

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